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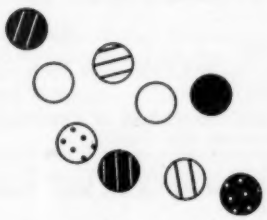

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An Intensive Study of Postvaccination Responses in Groups of Calves Vaccinated Intracutaneously and Subcutaneously with Strain 19 *Brucella Abortus* Vaccine

CORNELIA M. COTTON, Ph.D.

College Park, Maryland

THE USE OF intracutaneous vaccination as an immunizing agent for brucellosis was first reported by Cotton, Buck, and Smith [1] in a small number of cattle vaccinated with 0.5 ml. of three *Brucella abortus* vaccines. Different strains of *Brucella abortus* were used in the preparation of the vaccines, one of low virulence (strain 11), another of moderate virulence (strain 19), and the third of high virulence (strain 484). Since strains 19 and 484 became localized in the udder in the intracutaneously vaccinated animals, but not in those subcutaneously vaccinated, it was concluded that the danger of infecting milk and thus making vaccination a public health hazard was so great that the continuance of investigations on intracutaneous adult vaccination was not warranted.

Rabstein and Cotton [2] introduced vaccination of calves in field experiments with intracutaneous technique, using 0.2 ml. of strain 19 vaccine with good results. Since that work was reported, this technique has been used by several investigators. Campbell and Rodwell [3] reported that the intracutaneous vaccination produced high agglutination titers in cattle similar to those obtained when 25 times this dose was given subcutaneously. McDermid [4] found that intracutaneous vaccination of guinea pigs produced as satisfactory an immunity as did 25 times this dose, given subcutaneously. These observations were confirmed by McDermid [5] in heifers vaccinated at 15 to 18 months of age and, during the fifth month of pregnancy, infected with 150 million organisms.

MATERIALS AND METHODS

This experiment was conducted in the field under the best conditions available, somewhat simulating controlled laboratory experiments. All animals concerned were in six herds and all were dairy cattle of several breeds. About one half of the animals in the experiment in both groups were on farms with infected cattle, and the remainder were in accredited herds. All calves were separated from the adults after weaning.

Calves from 5 to 7 months of age were vaccinated intracutaneously and subcutaneously with strain 19 vaccine supplied by the Bureau of Animal Industry. One hundred and eight animals received 0.2 ml. of the vaccine in the skin of the left caudal fold or of the neck. The site of vaccination was changed to the neck to prevent any interference with future tuberculin tests. Another 108 animals were vaccinated subcutaneously with the usual 5 ml. of the vaccine.

Blood samples were drawn before vaccination and then daily from both groups of animals for fourteen days at monthly intervals for twenty-four months thereafter. Opsonocytophagic tests, as well as erythrocyte, leukocyte, and differential counts, were made on most of the samples in both groups in all pre- and postvaccination tests. All agglutination tests were made in dilutions of 1 : 25 to 1 : 2000. While some few of the calves developed titers greater than this, they were not considered particularly significant and are not included in these data.

Except for some slight modifications, the technique described by Huddleson [6] was used for the opsonocytophagic tests. Twenty-five neutrophils were examined and the results tabulated as follows:

Marked—40 or more bacteria per leukocyte.
Moderate—21 to 40 bacteria per leukocyte.
Slight—1 to 20 bacteria per leukocyte.
Negative—no bacteria per leukocyte.

A test was considered highly positive when 75 per cent of the leukocytes showed marked phagocytosis. Since many phago-

From the Live Stock Sanitary Service, University of Maryland, in coöperation with the Bureau of Animal Industry, U.S.D.A.

Acknowledgment is made to Dr. Melvin M. Rabstein for much of the earlier work done in this study.

cytes will normally engulf one or two Brucella, even a completely negative opsonocytophagic test will show some cells classified as "slight."

Experimental Data

PREVACCINATION STATUS OF ANIMALS

The animals in the intracutaneous group were negative to the agglutination test in the prevaccination bleeding, with the exception of 2 calves. These had been nursing positive dams and showed agglutination titers of 1:50 and high opsonocytophagic tests. Six other calves showed high opsonocytophagic reactions when tested before vaccination, and these had also nursed positive dams from birth. Opsonocytophagic indexes were virtually negative in all other calves. The averages were: marked, 2.0; moderate, 1.4; slight, 11.5; negative, 9.1 for all animals, including the 8 mentioned above.

The subcutaneous group contained 3 animals with titers of 1:25 or 1:50, the rest being negative. These 3, together with 5 others, had high opsonocytophagic indexes and all were known to have nursed positive dams. The average opsonocytophagic index in the subcutaneous group was: marked, 5.8; moderate, 3.8; slight, 9.0; and negative, 6.4. All animals, except the 8 referred to previously showed extremely low opsonocytophagic indexes. Prevaccination studies are shown in detail in tables 2 and 3.

POSTVACCINATION STUDIES

Agglutination Responses in Intracutaneously Vaccinated Animals.—A slight titer was present in a few of the animals within four days after vaccination; by the seventh day all calves, except the 2 showing prevaccination titers, were positive in dilutions of 1:50 or greater.

The average maximum titer developed was 1:980, but the individual titers ranged from 1:50 to 1:2000 and greater (fig. 1).

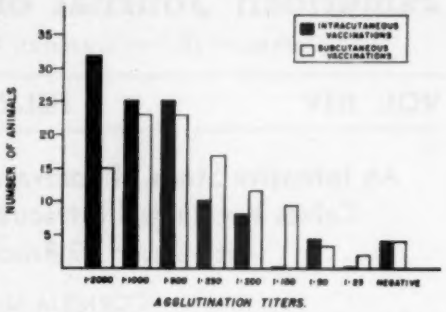

Fig. 1—Agglutination titers in 108 intracutaneously and 108 subcutaneously vaccinated calves two weeks following vaccination.

All animals had acquired their peak titers within eleven days after vaccination. Titers remained at their maximum for a week and gradually began to recede. By the end of two months, 70.3 per cent of the animals were negative; at four months, 85.6 per cent; and by the end of six months all were negative except 3 animals, which continued to carry fluctuating titers of 1:25 and 1:50 for the twenty-four months. Detailed results on titer retention are shown in table 1 and figure 2.

The group of 8 calves, which had high opsonocytophagic reactions prior to vaccination, were interesting in that they failed to develop high agglutination titers. The 2 animals which had titers of 1:50 before vaccination failed to respond with the production of higher titers, and both calves became negative within two months. The other 6 animals, showing high opsonocytophagic tests but no titers, responded to vaccination with the production of titers of

TABLE 1—Postvaccination Titers in 108 Intracutaneously and 108 Subcutaneously Vaccinated Animals

Agglutination titers (%)	Intracutaneously vaccinated						Subcutaneously vaccinated					
	Postvaccination titers						Postvaccination titers					
	Two Wk.	One Mo.	Two Mo.	Six Mo.	One Yr.	Two Yr.	Two Wk.	One Mo.	Two Mo.	Six Mo.	One Yr.	Two Yr.
Negative	3.7	28.7	70.3	97.2	97.2	97.2	5.5	7.4	18.5	64.8	81.4	79.6
1:25	1.8	17.6	9.2	1.8	0.92	1.8	7.4	7.4	7.4	9.7	7.4	9.2
1:50	3.7	11.1	6.4	0.97	1.8	0.92	2.7	7.4	11.1	16.6	7.4	6.4
1:100	90.7	42.6	13.8	0.0	0.0	0.0	89.7	77.7	62.9	7.4	3.7	4.6

Ages at Vaccination			
Intracutaneous		Subcutaneous	
No. animals	age	No. animals	age
14	4 mo.	18	4 mo.
20	5 mo.	17	5 mo.
56	6 mo.	59	6 mo.
18	7 mo.	14	7 mo.

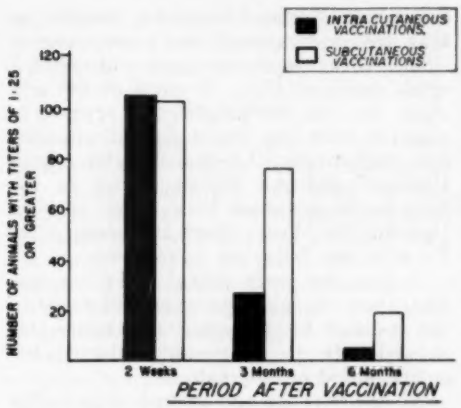

Fig. 2—Agglutination titers in two groups of intracutaneously and subcutaneously vaccinated calves at intervals following vaccination.

1:50 and 1:100. These titers were transitory and were lost within two weeks.

Agglutination Responses in Subcutaneously Vaccinated Animals.—The first signs of agglutination titers in the subcutaneously vaccinated calves appeared by the end of the fourth day after vaccination; two thirds of the animals were showing titers by the sixth day; and by the eighth day all had titers except the 8 which had shown high opsonocytophagic indexes prior to vaccination. The average titer of the subcutaneously vaccinated group was 1:670, but the range in this group was also 1:50 to 1:2000. Figure 1 shows a comparison of titers developed in intracutaneously and subcutaneously vaccinated animals. At four months, 26.1 per cent of the calves were negative, and at six months 64.8 per cent showed no titers. About 20 per cent of this group of the animals were still showing titers of 1:25 or greater at two years. Table 1 and figure 2 show the status of this group of animals over a two-year period.

Of the 8 animals with high opsonocytophagic indexes before vaccination, 5 never developed titers. The daily testing of the 3 remaining animals in the group, all of which had titers before vaccination, showed that they were negative the day following vaccination and for the three succeeding days, but titers reappeared on the fifth day at 1:50 and rose to 1:200 and 1:250 on the sixth day, then rapidly declined, so that at the end of one month these animals were completely negative.

Opsonocytophagic Responses in Intracutaneously Vaccinated Animals.—The first indication of a rise in the opsonocytophagic index in this group of calves occurred the third day after vaccination. Two thirds of the animals had reached the highest measurable index averaging marked, 23.5; moderate, 1.1; slight, 0.4; negative, 0.0, by the fifth day, and all had reached that point by the sixth day after vaccination. This average was maintained through the entire twenty-four months of testing with only very slight decreases. The 8 animals which had high opsonocytophagic tests before vaccination retained them from four to eight months following vaccination, after which they were rapidly lost.

Opsonocytophagic Responses in Subcutaneously Vaccinated Animals.—The first indication of positive opsonocytophagic tests appeared on the fourth day after vaccination, and the peak period began on the sixth day. The test showed that most of

TABLE 2—Average Indexes of the Pre- and Postvaccination Opsonocytophagic Tests in Intracutaneously and Subcutaneously Vaccinated Calves

	Intracutaneously vaccinated calves				Subcutaneously vaccinated calves			
	Marked	Moderate	Slight	Neg.	Marked	Moderate	Slight	Neg.
Prevaccination tests	2.1	2.5	11.5	8.9	5.8	3.8	9.0	6.4
Postvaccination tests								
First day	2.0	1.4	12.5	9.1	4.2	2.6	10.1	8.1
Second day	3.1	1.3	15.2	5.4	4.7	3.2	10.2	6.9
Third day	8.4	4.1	11.0	0.5	5.9	3.6	12.3	3.2
Fourth day	17.2	3.5	4.3	0.0	11.5	3.1	9.4	1.0
Fifth day	23.1	0.9	1.0	0.0	19.6	3.4	2.0	0.0
Sixth day	23.2	0.4	1.4	0.0	24.3	0.7	0.0	0.0
Seventh day	23.4	1.3	0.3	0.0	24.1	0.9	0.0	0.0
Eighth day	24.1	0.9	0.0	0.0	23.8	0.0	1.2	0.0
Fourteenth day	23.9	1.0	0.1	0.0	24.5	0.0	0.5	0.0
Sixth month	21.1	2.3	1.6	0.0	20.2	0.8	4.0	0.0
Twelfth month	19.7	4.2	1.1	0.0	20.6	2.3	2.1	0.0
Eighteenth month	17.8	5.2	2.0	0.0	16.8	6.3	1.9	0.0

the animals maintained their high opsonocytophagic indexes for twenty-four months, but during the last six months there was a gradual decrease in measurable opsonins. The 8 calves with high opsonocytophagic indexes prior to vaccination followed the same postvaccination course as did those in the intracutaneous group. Comparative studies of the opsonocytophagic response are given in table 2.

BLOOD PICTURE

The erythrocyte count varied so little following vaccination that it will be completely ignored in this discussion. The white blood count showed marked variation from the day following vaccination and for some time thereafter.

The Blood Picture in Intracutaneously Vaccinated Animals.—On the day following vaccination, the average leukocyte count had greatly increased because of the production of neutrophils, which averaged 3,753 before vaccination and increased to an average of 7,315 within twenty-four hours. In individual cases, the increase in numbers of neutrophils was much more marked than this average would indicate.

By the fourth day, the average leukocyte count had dropped somewhat, due to a great reduction in the number of neutrophils, but there was a considerable rise in both the percentage and the actual number of lymphocytes. In some individuals, the lymphocyte count was as much as 95 per cent of the total white cell count. On the eighth day, the average total leukocyte count was decreased somewhat further, so that it approximated the prevaccination count. However, the proportion of lymphocytes remained high. In most of the animals, the rise in lymphocytes seemed to coincide with the production of opsonins and agglutinins. Lindquist, Zehner, and Phillips [7] did not report a rise in the lymphocyte count in their work, nor did they find the blood pattern influenced after the fifth day following vaccination.

During the early period after vaccination, there was no appreciable difference in the eosinophils. Basophils and monocytes showed little or no variation during the entire period of observation.

At the end of the second month, the average leukocyte count was slightly higher than it had been at eight days, but the percentages of lymphocytes and neutrophils had returned to their prevaccination relationships. At this time, there was a marked increase in the number of eosinophils in all animals, some showing as high as 12 per cent. This increase seemed to coincide with the disappearance of the agglutination titers in many of the animals. A summary of the blood studies is given in detail in table 3.

The Blood Picture in Subcutaneously Vaccinated Animals.—The blood studies in this group of animals varied little from the intracutaneously vaccinated group. The same changes were noted and the averages in the day-to-day study were about the same in both groups (table 3). A rise in the eosinophils was not noted in many

TABLE 3—Blood Studies of 108 Intracutaneously and 108 Subcutaneously Vaccinated Calves

		Average number blood corpuscles per cmm. blood					
		Prevaccination test	Postvaccination test				
			1st Day	4th Day	8th Day	2nd Month	3rd Month
Intracutaneously vaccinated calves	Total leukocytes	11,137	14,631	13,653	11,550	15,100	11,400
	Neutrophils	3,753	7,315	3,276	2,156	4,530	3,648
	Lymphocytes	6,781	6,662	9,654	8,740	9,266	6,999
	Monocytes	441	485	546	444	604	456
	Basophils	33	23	27	29	36	35
	Eosinophils	129	146	150	181	664	262
Subcutaneously vaccinated calves	Total leukocytes	12,170	15,128	13,958	12,908	13,256	12,630
	Neutrophils	3,900	7,362	3,348	2,193	2,408	2,828
	Lymphocytes	7,517	6,915	9,735	9,837	9,856	8,932
	Monocytes	467	475	535	516	505	432
	Basophils	121	75	102	106	117	129
	Eosinophils	265	301	238	256	370	309

individuals during the second month, but this may have been because there was such a wide variation in the time of titer loss.

Discussion

The vaccination of calves which have been nursing positive dams presents an interesting problem. In the work reported here, as well as in other experiments at this station, it would appear that sufficient immunity develops in calves allowed the colostrum and milk of positive dams to reduce the efficacy of calfhood vaccination to a very considerable degree. Such calves were found to have unusually high opsonocytophagic indexes and, in some cases, titers before they were vaccinated. In every case, it was possible to identify these animals through their opsonocytophagic indexes without first knowing their previous history.

It has been noted in quite a number of instances that such animals were very susceptible to brucellosis and contracted the disease regularly when exposed naturally at 2 to 3 years of age. Four animals in this experiment acquired brucellosis at 2½ years of age from natural exposure.

Summary

1) Agglutination and opsonocytophagic tests and some hematological studies were made the basis of comparison of intracutaneous and subcutaneous vaccination of calves between the ages of 5 and 7 months. Strain 19 *Brucella abortus* vaccine was used in both groups with 0.2 ml. inoculated intracutaneously and 5.0 ml. subcutaneously.

The two groups of 108 calves each showed the same prevaccination status. There were 8 animals in each group which had nursed positive dams and as a result had high opsonocytophagic indexes. Five of the above-mentioned 16 showed agglutination titers of 1 : 25 and 1 : 50.

2) The production of opsonins and agglutinins in intracutaneously vaccinated calves was at least equal to, and in many cases greater than, those produced in subcutaneously vaccinated animals where 25 times the dose of vaccine was used.

3) Agglutinins in both groups were measurable on the fourth day after vaccination, and by the tenth and eleventh days titers had reached their peak. Two months following intracutaneous vaccination, 70.3 per cent of the calves were negative, while 18.5 per cent of those subcutaneously vaccinated were without titers. At the end of six months, 97.3 per cent of the intracutaneously vaccinated heifers were negative, but only 64.8 per cent of those vaccinated subcutaneously had no titers.

4) A marked rise in the opsonins was noted the third and fourth day after vaccination in the two groups. On the fifth day, the intracutaneously vaccinated animals, and on the sixth day those subcutaneously vaccinated, showed the highest measurable peak by the Huddleson technique. The opsonocytophagic indexes of these animals remained high for eighteen months and then began to decline very slowly.

5) Exceptions to the above pattern occurred in the 16 animals which had nursed positive dams. This group developed slight transitory titers or remained negative as a result of the vaccination and appeared to be susceptible to brucellosis at 2½ years of age, when 4 of them acquired the disease from natural exposure.

6) Hematological studies proved to be very similar in the two groups. Variations in the erythrocyte counts were almost negligible. A rise in the leukocyte count within twenty-four hours proved to be due to an increase in the neutrophils. The white cell counts were reduced within forty-eight hours due to a decline in neutrophils, but a marked increase was noted both in percentage and actual number of lymphocytes. This lymphocytosis persisted for seven to eight days, after which the blood picture returned to nearly normal. The production of opsonins or agglutinins seems to stimulate, or at least to coincide with, a rise in lymphocyte counts. With the loss of titer, there appears to be an increase in the number of eosinophils, especially noticeable in the intracutaneously vaccinated animals. Other blood cells did not vary, especially in number or morphology, at any time following vaccination.

References

[1] Cotton, W. E., Buck, J. M., and Smith, H. E.: Efficacy and Safety of Abortion Vaccines Prepared from *Brucella Abortus* Strains of Different Degrees of Virulence. J. Agric. Res., *46*, (1933) : 291-314.

[2] Rabstein, Melvin M., and Cotton, Cornelia: Bang's Disease Immunity Tests and a New Vaccination Method. Proc. 46th meet. U. S. Livestock San. A., *98*, (1942) : 129-136.

[3] Campbell, A. D., and Rodwell, A. W.: The Relationship of Dosage and the Site of Inoculation to the Agglutination Response to *Brucella Abortus* Strain 19 Vaccine: A Comparison of Subcutaneous, Intracutaneous, and Intracaudal Routes. J. Comp. Path. and Therap., *55*, (1945): 277-289.

[4] McDermid, A.: A Comparison of the Immunity Produced in Guinea Pigs by the Inoculation of S-19 *Brucella Abortus* Vaccine Intradermally and Subcutaneously. Vet. Rec., *60*, (1948): 227-228.

[5] McDermid, A.: A Comparison of the Immunity Produced in Cattle by the Inoculation of *Brucella Abortus*, Strain 19, Intradermally, Intracaudally and Subcutaneously. Vet. Rec., *62*, (1950): 361-364.

[6] Huddleson, I. F., Johnson, H. W., and Meyer, D. B.: A Method for Measuring the Opsonocytophagic Power of the Blood of Cattle for Brucella. Mich. Agric. Exp. Sta. Tech. Bull. *149*, 1936.

[7] Lindquist, Norman B., Zehner, C. Earnest, and Phillips, Paul H.: The Effect of Vaccination with *Brucella Abortus* Vaccine on Certain Blood Constituents in Young Heifers. J. Dai. Sci., *33*, (1949): 914-918.

The Pathology of Experimental Hypovitaminosis A in Young Dairy Animals

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HYPOVITAMINOSIS A in dairy cattle has been considered to be the cause of a wide variety of clinical and pathological alterations. A report by the National Research Council in 1950 listed such changes as follows:[29] roughened coat, dry pityriasis, lacrimation, keratitis, corneal softening, xerophthalmia, nyctalopia, blindness, emaciation, anorexia, diarrhea, incoördination, convulsions, and pneumonia. In addition to the foregoing symptomatic manifestations, mention was made of increased cerebrospinal fluid pressure, epithelial metaplasia in the respiratory tract, buccal cavity, salivary glands, eyes, lacrimal glands, intestinal tract, urethra, kidney, and vagina. Such a formidable array of changes could have contained nonspecific alterations. In this connection, Follis[12] emphasized the importance of differentiating between specific and nonspecific tissue damage owing to the deficiency of a particular substance.

The present study was concerned with a pathological analysis of young dairy animals which had rapidly been reduced to a state of vitamin A deficiency in order to eliminate nonspecific changes as far as possible.

REVIEW OF LITERATURE

The symptoms of vitamin A deficiency were recognized in Biblical times about 601 B.C., according to Langham[25] who quoted an Old Testament verse to the effect that wild asses lost their vision owing to a shortage of grass.

Subcutaneous edema (anasarca) was reported in the feeder cattle industry.[5, 60] When anasarca was reproduced experimentally, two years were required in one instance,[45] although shorter periods were also reported.[5, 58] Histological studies[6] of a natural outbreak revealed an edematous process accompanied by infiltration with polymorphonuclear leukocytes, plasma cells, lymphocytes, histiocytes, and erythrocytes. Involved muscles lost striations and became hydropic. The vascular intima became hyperplastic and the nerves atrophic.

Ocular changes have been ascribed to vitamin A deficiency. Keratitis,[39] corneal ulceration,[15] hair growth on the cornea,[45] and xerophthalmia have been seen.[22, 25] Nyctalopia[25, 42] and total blindness[11, 12, 19, 30, 42] were of common occurrence. Ophthalmoscopic examination revealed fading of the tapetum lucidum, papilledema,[5, 39] and retinal hemorrhages.[5, 11] The optic nerves appeared constricted[30] at the point where they passed through the optic foramen, whose lumen was narrowed[30] owing to intracranial pressure. This increase of intracranial pressure was attributed to perivascular edema[9] and in the case of the rat to actual overgrowth of the brain.[39] The increase of this pressure as caused by A avitaminosis was determined by measuring the cerebrospinal fluid pressure in dairy calves at the atlanto-occipital joint with the aid of a saline mannometer.[60] Normal cerebrospinal fluid pressure was in the vicinity of 105 mm.,[60] but rose as high as 600 mm. in deficient calves.[58]

The pituitary gland underwent two types of changes. Cystic degeneration was frequently reported[11, 18, 30, 37, 51, 55] and appeared to result from dilation of the residual lumen of Rathke's pouch. The other phenomenon was the so-called castration effect or decrease of basophilic cells in the glandular portion as reported in the rat deficient in vitamin A.[35, 49] It was postulated that the castration effect resulted from a decrease in pituitary secretion rather than of lack of androgen inasmuch as the administration of testosterone caused a hypertrophy of the accessory sexual glands in deficient rats.[35, 60] In vitamin A-deficient calves, an increase of acidophilic cells[40] was seen and was believed to be similar to the basophilic increase in rats.

The effects of vitamin A deficiency on the thyroid gland were studied chiefly in rats and the results were contradictory. There were reports that the thyroid was unaffected[41, 44, 47] while other authors noted hypertrophy of the gland in male rats and atrophy in the female rat.[4] Histological changes were variously described as atrophy with epithelial degeneration,[7] hyperplastic epithelium,[39] and follicles full of colloid with flattened epithelium.[60] That vitamin A had some relation to the thyroid gland was demonstrated in that thyroidectomized rats stored large amounts of vitamin A as contrasted to the low storage in normal controls.[17] However, uptake of I^{131} was similar in both normal and deficient rats.[20]

Gastrointestinal disturbances were noted by the objective symptom of diarrhea.[9, 10, 15, 16, 23, 26, 35, 42]

From the Department of Animal Diseases and Animal Industries, Storrs Agricultural Experiment Station, University of Connecticut, Storrs, and the Bureau of Dairy Industry, U.S.D.A.

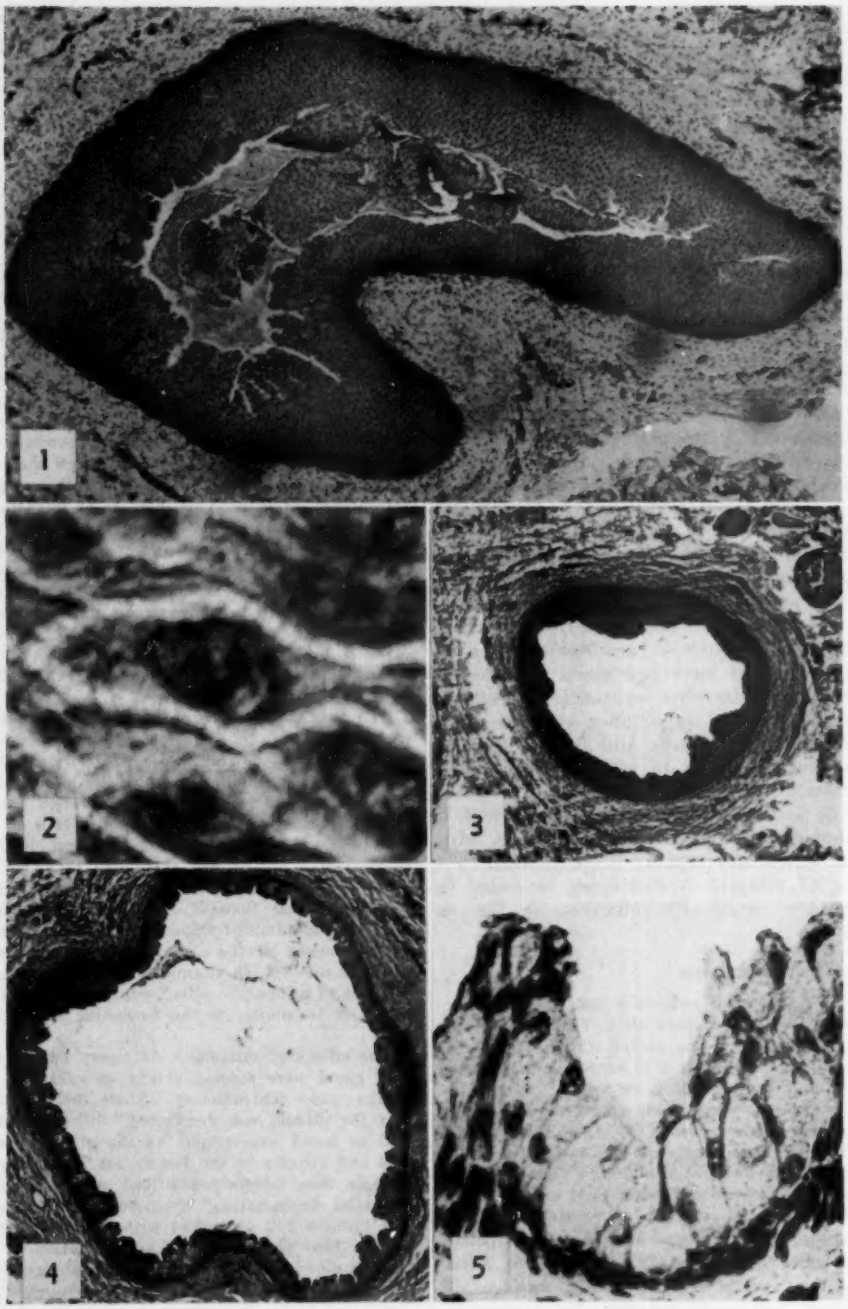

Plate 1

Photomicrographs of sections fixed in formalin, imbedded in paraffin, and stained with hematoxylin triosin.

Histological examination revealed swollen intestinal mucosa with eosinophilic and lymphocytic infiltration of lamina propria, edematous mesenteric lymph nodes, cloudy swelling and fatty degeneration of the liver with focal necrosis.[61]

The respiratory tract was frequently affected, with pneumonia being the usual result.[15, 20, 25, 54]

The kidneys in vitamin A-deficient calves have presented gross alterations consisting of a mottling[25] termed "white spotted kidney,"[37] owing to accumulations of lymphocytes and macrophages.[61] Tubular changes consisted of epithelial alterations such as parenchymatous and hydropic degeneration, or actual necrosis[26] particularly of the proximal convoluted tubuli.[61] Interstitial fibrosis and lymphocytic foci with thickening of Bowman's capsule were common changes,[61] whereas metaplasia or keratinization of the tubuli[26] was seen rarely.

Renal calculi have been ascribed to a deficiency of vitamin A and produced to some extent experimentally in rats[60] and foxes.[1] Calculi formation in cattle was not a common finding in vitamin A deficiency as little mention was made of it in the literature.[45]

Reports of the effect of vitamin A deficiency on the bovine testes were ambiguous. For example, it was reported that viable spermatozoa decreased while libido and spermatozoal concentrations were normal.[3] Other papers reported low semen quality,[27] depressed libido,[11] and lowered spermatozoal concentrations.[58] It was suggested that spermatogenesis failed to occur if vitamin A deficiency was present prior to or at puberty but that spermatogenesis continued if already established.[45] Histological examination revealed degeneration and necrosis of the seminiferous epithelium.[3, 22, 51]

Squamous metaplasia has been termed the cardinal sign of vitamin A deficiency.[66] Wolbach and Howe[67] first reported that stratified keratinized epithelium replaced the normal types in the respiratory tract, eyes, alimentary tract, and genitourinary tract of vitamin A-deficient rats. This replacement arose from the multiplication of the basal epidermal cells while the normal epithelium atrophied.[39] The nasal passages of the domestic fowl reacted in a like manner when a deficiency of vitamin A existed.[20, 49] Squamous metaplasia in the interlobular ducts of the parotid gland was first reported by Jungherr *et al.*[21, 22] in calves deficient in vitamin A. Another investigator[25] saw the process in the bronchi, mandibular salivary glands, and in the cervix of the uterus. In the last organ, squamous metaplasia was one of the features of hyperkeratosis,[14, 24] but the fact that hyperkeratosis produced a concomitant hypovitaminosis A as demonstrable by low blood plasma vitamin A levels[14] suggested that the lesions were the result of the vitamin deficiency.

Low blood plasma vitamin A levels have been reported to produce characteristic evidence of deficiency. Seven to 8 μg. of vitamin A per 100 cc. of plasma were considered to be on the borderline[2] and 10 μg. per 100 cc. necessary for growth. Expressed in the terms of plasma carotene, it was found that symptoms commenced when carotene fell below ten μg. per 100 cc.[54] Eaton *et al.*[9, 10] consistently produced squamous metaplasia when the blood plasma vitamin A fell to 4 μg. per 100 cc.; cystic pituitary glands occurred at the same level.[18]

Materials and Methods

Fifty-four dairy calves of the Holstein-Friesian and Guernsey breeds constituted the animals employed in this project. All but 4 were males and, unless otherwise indicated, were 105 days old at the start of the experiments. The calves were at first raised on a limited whole milk, dry calf starter, and hay regimen, then subjected to the experimental treatment stated below and slaughtered at the end of the experiments. The experimental groups, as detailed below, were made up primarily for the study of the biochemical effects of vitamin A depletion but formed excellent material for pathological investigation on account of their known nutritional history.

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Plate 1

Fig. 1—Main parotid duct from a 166-day-old Holstein-Friesian male calf with a blood plasma vitamin A level of 1.2 μg. per 100 cc. There is marked squamous metaplasia of the epithelium. x 45.

Fig. 2—Portion of the main parotid duct from a 190-day-old Guernsey male calf with a blood plasma vitamin A level of 2.2 μg. per 100 cc. The intercellular bridges, characteristic of squamous metaplasia, are seen in the metaplastic epithelium. x 2362.

Fig. 3—An interlobular duct of the parotid gland from the same calf as in figure 2 showing moderate squamous metaplasia. x 30.

Fig. 4—Main parotid duct from a 182-day-old Holstein-Friesian male calf with a blood plasma vitamin A level of 1.6 μg. per 100 cc. At 10 o'clock there is a patch of metaplastic epithelium while the remaining epithelium is the normal columnar vacuolated type. x 30.

Fig. 5—Main parotid duct from a 94-day-old Holstein-Friesian male calf with a blood plasma vitamin A level of 2.0 μg. per 100 cc. In this area normal pseudostratified columnar epithelium still persists. x 510.

Control Group 1.—Six individuals were placed on a vitamin A-depletion ration * fed at the rate of 2.67 lb. per 100 lb. of body weight adjusted at seven-day intervals,[30] plus 107,143 USP units of vitamin A in the form of shark liver oil.

Depleted Group 2.—Nineteen 105-day-old animals were fed the depletion diet.

Depleted Group 3.—Five 63-day-old calves were fed the depletion diet.

Depleted animals were killed when the blood plasma vitamin A level reached 4 μg. per 100 cc. or less for two consecutive weeks. Control animals were slaughtered at the same time as the depleted animals.

Carotene-Treated Group 4.—Four calves were fed the depletion diet until blood plasma vitamin A was less than 4 μg. per 100 cc. for fourteen days, followed by a six-day standardizing period, and a five-day period of intravenous administration of the dispersing agent (9 % "tween" 80). After an additional five-day term of observation, the animals received crystalline *beta* carotene** at the rate of 120 μg. per pound of body weight in the dispersing agent daily for six days and then were slaughtered after a lapse of fourteen days.

Carotene-Treated Group 5.—Treatment was the same as for group 4, except carotene and dispersing agent were given *per os.*

Treated Group 6.—Six calves were fed on the depletion ration until blood plasma vitamin A was less than 4 μg. per 100 cc. for fourteen days, at which time they were treated daily for fourteen days with 200,000 U.S.P. units of vitamin A administered *per os.*

Treated Group 7.—Four calves were fed as in group 6 but given vitamin A for four weeks.

Treated Group 8.—Six calves were handled as group 6 but given 2 Gm. of ascorbic acid daily for fourteen days, administered subcutaneously.

Blood plasma vitamin A and carotene were determined by Kimble's[24] method, liver vitamin A and carotene by Davies'[6] method, and ascorbic acid by Roe's[40] procedure. The cerebrospinal fluid pressure was determined by the method of Sykes and Moore.[50] Values in all cases were determined when the depletion diet was started, and at time of slaughter. In addition, the same determinations were also taken before ascorbic acid, carotene, or vitamin A was administered.

Sections for histopathological study were taken of the pituitary gland, parotid gland and duct, submaxillary salivary gland, adrenal gland, colon, eye, heart, liver, lung, small intestine, thyroid gland, testis, or cervix, ovary, and udder. Formalin was the usual fixative although Bouin's was often employed for the endocrine glands. The tissues were imbedded in paraffin and stained with Harris-hematoxylin and triosin. In addition, Masson's trichrome was used to stain the pituitary gland.

* A mixture by weight of one third of dried beet pulp and two thirds of a grain mixture composed of the following: 419.5 lb. ground barley, 500 lb. crimped oats, 500 lb. wheat bran, 150 lb. linseed oil meal (expellar process), 150 lb. soybean oil meal (expellar process), 200 lb. cane molasses, 40 lb. 500 potency B-Y dried fermentation solubles, 20 lb. steamed bone meal, 20 lb. iodized salt, and 0.5 lb. irradiated yeast (Standard Brands type 9-F) per ton of mixture. This depletion ration contained 0.25 mg. of carotene per pound.

** Crystalline *beta* carotene, General Biochemical, Inc., Chagrin Falls, Ohio.

RESULTS

Vitamin A deficiency, as measured by a blood plasma level of 4 μg. per 100 cc. of vitamin A, consistently produced squamous metaplasia of the parotid gland main duct and interlobular ducts. The main duct was the most sensitive tissue (table 1). Of the 24 calves with lowered blood plasma vitamin A, 23 showed squamous metaplasia of the main parotid duct (fig. 1 and 2) while 16 showed lesions in the interlobular ducts of the parotid gland itself (fig. 3).

Normally, the interlobular ducts and the main parotid ducts were found to be lined by pseudostratified columnar epithelium and often to contain light-staining colloid material near the free border (fig. 4 and 5), particularly in the main duct. At the oral termination of the parotid duct, known as the papilla, there was a poorly defined but recognizable zone representing the mucocutaneous junction between the columnar epithelium of the main duct and the normal squamous epithelium of the oral cavity (fig. 6). A more abrupt demarcation was reported [20] in the nasal sinuses of the domestic fowl (fig. 7 and 8). When birds became deficient in vitamin A, the mucocutaneous junction was either displaced dorsally or erased entirely owing to substitution of squamous epithelium for the normal respiratory epithelium; this was considered a specific lesion of vitamin A deficiency. In the dairy calf, the pseudocolumnar lining of the parotid main duct and interlobular ducts was likewise replaced by stratified squamous epithelium. The metaplastic process began at the basement membrane and progressed outward, generally altering the whole mucosa although remnants of the normal epithelium frequently persisted (fig. 9). The pathological process often resulted in the formation of cellular bridges and microscopic polyps, and on the whole was unequivocal. In no instance were salivary calculi seen nor was there any evidence to suggest calculi formation.

Since the main duct of the parotid gland

TABLE 1—Effect of Vitamin A-Deficient Diet and of Various Dietary Supplements on Dairy Calves

Group	No. of calves	Age at slaughter (days)	Spinal fluid pressure (mm.)			Vitamin A assay				Carotene assay				Clinical symptoms	Histopathological changes								
						liver (γ/gm.)	blood plasma (γ/gm.)			liver (γ/gm.)	blood plasma (γ/gm.)				Kidney (IN)	Liver (FN)	Lung (PN)	Pituitary (cyst)	squamous metaplasia: Parotid duct	squamous metaplasia: Parotid gland	squamous metaplasia: Salivary gland	Testis (S)	Thyroid
			BD	D	T	TR	BD	D	T	TR	BD	D	T										
Deficient diet plus 107,143 U.S.P. units of vitamin A daily (controls)																							
1	6	177	95		119	155.0			38.2	0.4			3.3		2	—	—	—	—	—	—	1	—
Deficient diet																							
2	19	177	95	330		0.3	25	2.5		0.4		6		5 Diarr. 9 Incoörd.	4	5	2	—	18	14	4	4	—
3	5	105		203		0.1	21	1.0		0.2		1		1 Diarr. 4 Incoörd.	—	1	3	—	5	2	1	—	—
Deficient diet plus carotene																							
4	4	216	106		360	0.2	26	2.0	5.0	1.0		7	41.0	2 Diarr. 2 Incoörd.	—	1	2	1	4	3	2	1	—
5	4	210	95		418	0.1	24	2.0	3.0	0.5		7	9.0	4 Diarr. 2 Incoörd.	—	1	3		4	4	3	—	—
Deficient diet plus vitamin A (therapeutically)																							
6	6	191	95	220	159	34.0	23	2.0	40.0	1.0		8	5.0	—	—	1	—	—	3	—	1	—	—
7	4	194	95	380	118	126.0	24	2.0	37.0	1.0		4	2.0	—	—	—	—	—	—	—	—	2	—
Deficient diet plus ascorbic acid																							
8	6	184	95	211	269	0.1	22	2.0	2.0	0.3		7	6.0	3 Diarr.	2	1	2	—	6	4	4	2	—

BD—before depletion; D—depleted; FN—focal necrosis; IN—interstitial nephritis; PN—pneumonia; S—spermatogenesis; T—treated; TR—terminal value; blank space—not done; — —normal.

Details for various groups will be found under "Materials and Methods."

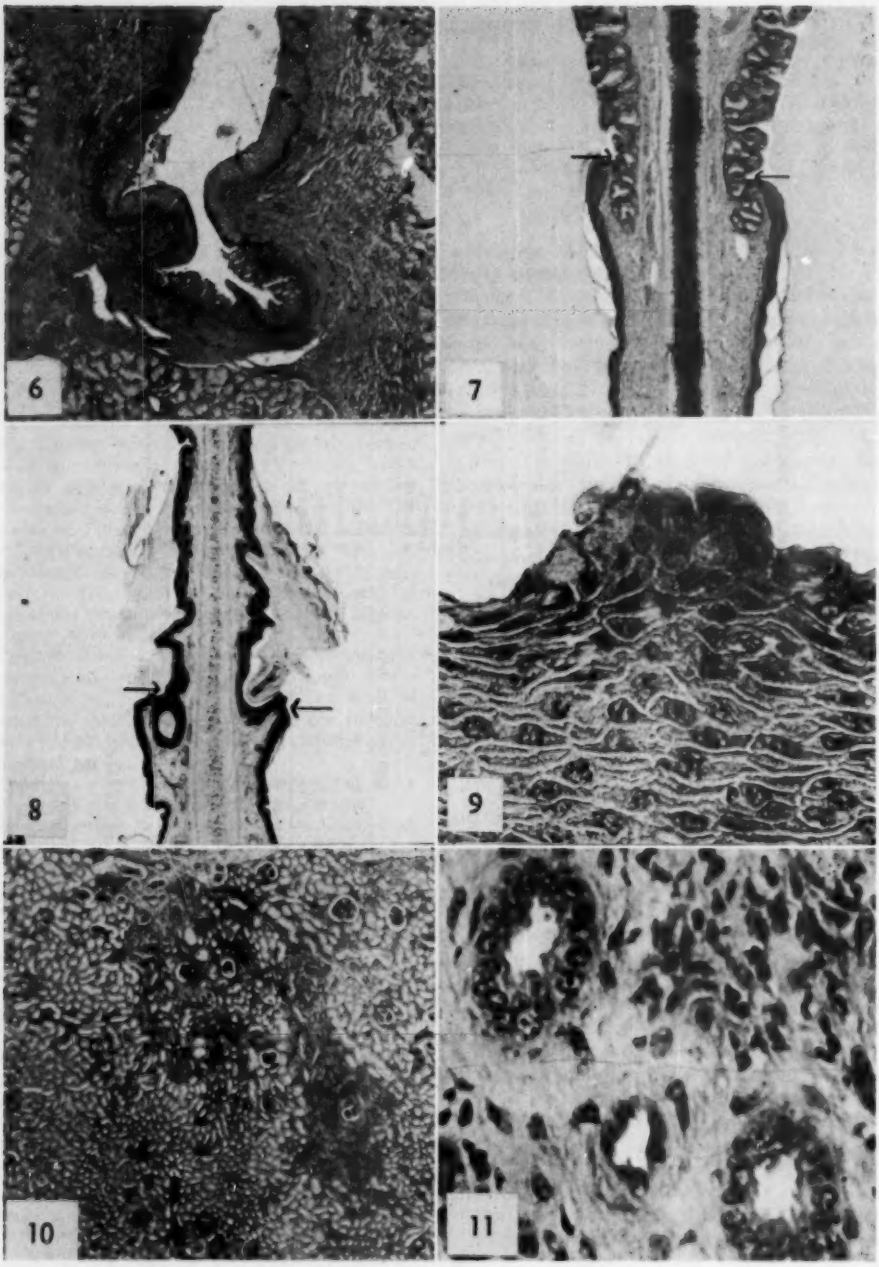

Plate 2

Photomicrographs of sections fixed in formalin, imbedded in paraffin, and stained with hematoxylin triosin.

and interlobular ducts proved specifically sensitive (table 1) these lesions were used as a criterion in the evaluation of other signs and lesions ascribed to hypovitaminosis A. As shown in table 1, the lesion of squamous metaplasia likewise occurred in other salivary glands, although not so frequently as in the parotid gland.

Although ophthalmoscopic examinations were made at frequent intervals throughout the lives of the various calves, no definite correlation could be ascertained between calves deficient in vitamin A and papilledema, except in marked examples of the lesion. It was likely that inexperience of the diagnostician (CFH) contributed to the above failure; the more delicate alterations were probably overlooked.

With reference to the symptoms observed, incoördination, diarrhea, and exophthalmus were the only ones seen and occurred in the frequency listed (table 1). The degree of incoördination varied from a staggering gait to convulsive movements in which the animal fell to the ground. In a previous report,[21] an animal was recorded which actually died while so affected.

The cerebrospinal fluid pressure proved to be a sensitive measurement of vitamin A deficiency. In calves prior to depletion and in experimentally supplemented calves, the pressure consistently was less than 95 mm. In depleted animals, the pressure rose up to 505 mm. in some instances and ordinarily was over 178 mm. (table 1).

Kidney alterations were frequently encountered in these experiments but did not appear to be related to vitamin A deficiency (table 1). Cortical interstitial foci of lymphocytes with or without fibrosis (fig. 10) were the usual lesions, although a condition believed to be benign nephrosclerosis was seen in 1 nondeficient calf (fig. 11). In no instance were the interstitial foci of such magnitude as to cause "white spotted kidney." The degenerative and necrotic epithelial changes reported in the literature, generally failed to appear in these rapidly depleted calves except in 1 instance (fig. 12). Renal calculi, either of gross or microscopic dimensions, were not observed although microscopic calculi were seen in the medullary portion of the kidney in 2 of 12 deficient sheep. In an unpublished experiment, 2 nondepleted calves which were serving as normal controls were found to contain countless calculi in both kidneys. Thus it appeared that hypovitaminosis A, when produced rapidly, did not cause lesions in the kidney.

Of the digestive tract, the liver alone presented changes. Focal necrosis appeared in 6 of the 24 depleted animals and in 4 of 24 animals which had been depleted but then treated with vitamin A, carotene,

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Plate 2

Fig. 6—A longitudinal section of the oral opening of the parotid duct from a 189-day-old Holstein-Friesian male calf with a blood plasma vitamin A level of 40.1 μg. per 100 cc. The upper lumen is delimited by normal stratified squamous epithelium whereas the lower presents columnar vacuolated epithelium. Arrow points to mucocutaneous junction. x 30.

Fig. 7—Nasal septum from a 28-day-old White Leghorn chicken fed a diet supplemented with vitamin A. The normal mucocutaneous junction is indicated by an abrupt change from stratified squamous to respiratory epithelium (arrow). x 30.

Fig. 8—Nasal septum from a 28-day-old White Leghorn chicken fed a diet deficient in vitamin A. The respiratory epithelium has been completely replaced by the squamous metaplastic epithelium. Arrow points to mucocutaneous junction. x 30.

Fig. 9—Portion of the main parotid gland duct from a 190-day-old Guernsey male calf with a blood plasma vitamin A level of 2.2 μg. per 100 cc. A tuftlike remnant (in high center) of normal epithelium rests on the squamous metaplastic epithelium. x 510.

Fig. 10—Kidney from a 108-day-old Holstein male calf with a blood plasma vitamin A level of 0.4 μg. per 100 cc. There is a large area of interstitial fibrosis slightly infiltrated by lymphocytes. x 30.

Fig. 11—Kidney from a 178-day-old Guernsey male calf with a blood plasma vitamin A level of 43.1 μg. per 100 cc. showing an increase of interstitial connective tissue suggestive of benign nephrosclerosis. x 825.

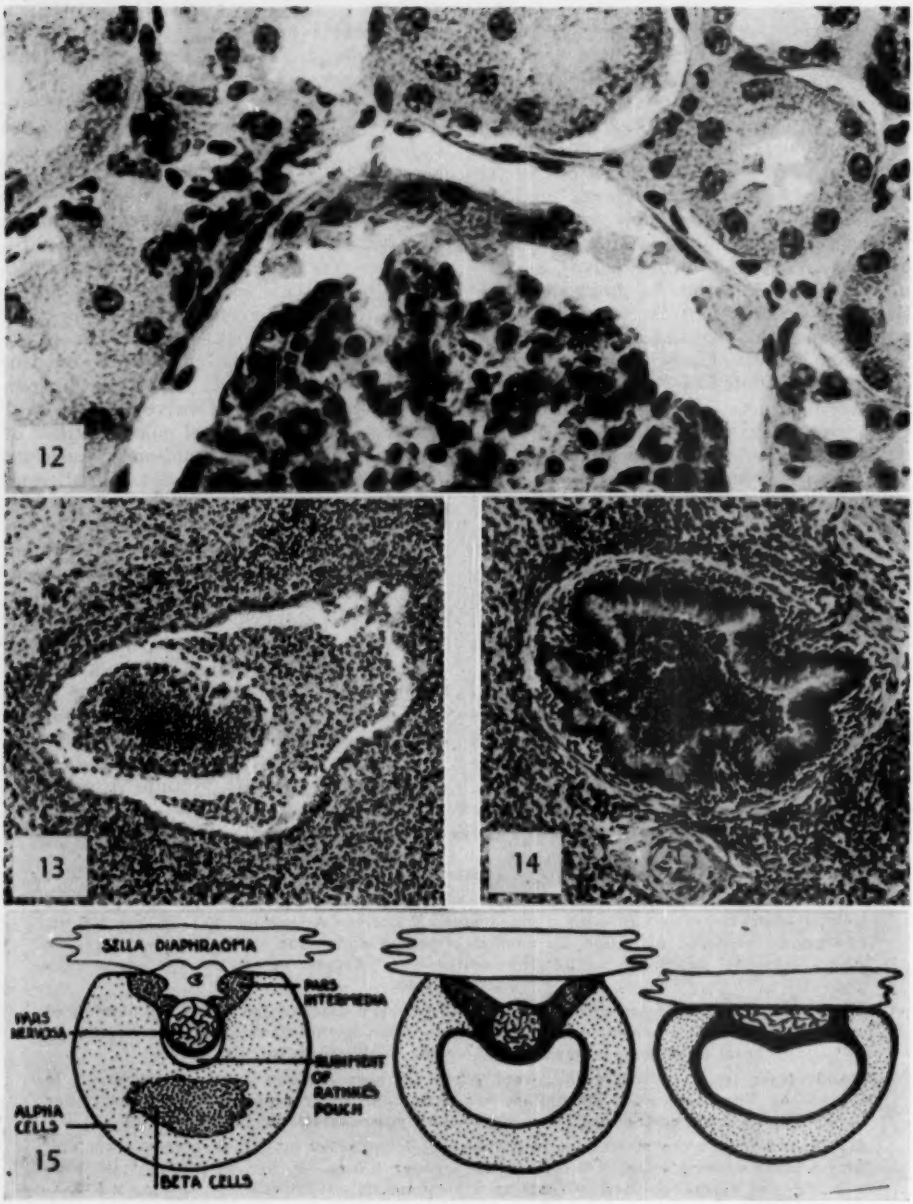

Plate 3

Photomicrographs of sections fixed in formalin, imbedded in paraffin, and stained with hematoxylin triosin.

or ascorbic acid. The lesion never appeared in the controls (table 1). The usual focus consisted of hepatic cells which had undergone cytolysis and pyknosis, and infiltration by lymphocytes. In many instances, the bile duct was involved in the process. A periductal infiltration by round cell elements with the lumen plugged by sloughed and phagocytic cells was common (fig. 13). Other portions of the digestive tract such as the small intestine and colon were without change. The existence of abnormally fluid feces termed diarrhea did not recognizably influence the histology of the intestinal mucosa.

Pneumonia occurred in 12 animals, all of which had been depleted. Seven had been treated by carotene or ascorbic acid (table 1). Ordinarily the pneumonia was focal in character, grossly suggesting a granuloma. Numerous heterophils filled the alveoli and bronchioles (fig. 14); fibrous proliferative areas often walled off the exudative process in an abrupt manner. There was no clinical evidence of the pulmonary condition such as dyspnea, coughing, or rise in temperature. The crude data suggested that depleted calves were more susceptible to pneumonia than supplemented animals. However, treatment of the data by the *chi*-square method failed to reveal a significant relationship between pneumonia and vitamin A depletion.

The pituitary gland failed to show cystic degeneration consistently. One cystic pituitary appeared to result from dilation of the residual lumen of Rathke's pouch (fig. 15). The cyst increased in size cephalocaudally; it was in the normal direction of the residual lumen.[19] The glandular portion often contained many so-called sinusoids [13, 52] which by their large size and relative frequency suggested multiple microcysts. Inasmuch as sinusoids were observed in animals of every group, they were considered to represent normal anatomic variations.

The castration effect reported in rats was not observed in calves. Midfrontal or sagittal sections of the pituitary glands consistently revealed about 70 per cent *alpha* cells, regardless of the availability of vitamin A. Parasagittal sections and anterior or posterior frontal sections revealed lesser numbers of *beta* cells. This variation occurred without relation to vitamin A intake. Since growth was generally normal in all experimental animals, the constancy of the percentage of *alpha* cells was not surprising as they have been considered as the source of the growth hormone.[13]

Measurement of depressed spermatogenesis was not possible inasmuch as calves were just approaching puberty when slaughtered. In a few instances, the seminiferous tubules contained a few primary spermatocytes, but such instances were sporadic and apparently not related in any way to vitamin A deficiency.

The thyroid gland presented no recognizable pathological alterations. The mild hyperplasia of the acinal epithelium reported [22] was not apparent in this study.

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Plate 3

Fig. 12—Kidney from a 184-day-old Holstein-Friesian female calf with a blood plasma vitamin A level of 8.4 μg. per 100 cc. Bowman's glomerular space contains loose epithelial cells (in center). x 510.

Fig. 13—Liver from a 182-day-old Holstein-Friesian male calf with a blood plasma vitamin A level of 8.2 μg. per 100 cc. The bile duct presents phagocytic cells and periductal infiltration. x 118.

Fig. 14—Lung from a 110-day-old Holstein-Friesian male calf with a blood plasma vitamin A level of 0.9 μg. per 100 cc. A bronchus exhibits heterophils in lumen and peribronchial infiltrates with the same cell type. x 118.

Fig. 15—Diagrammatic histoanatomy of bovine pituitary: Left—Cross section at middle from a 168-day-old Holstein-Friesian male calf with a blood plasma vitamin A level of 0.6 μg. per 100 cc. This is identical with sections taken from animals on high blood levels of vitamin A. Center—Cross section at middle from a 182-day-old Holstein-Friesian male calf with a blood plasma vitamin A level of 2.1 μg. per 100 cc. The residual lumen of Rathke's pouch is markedly dilated. Right—Same calf as center diagram but cross section near caudal end. The residual lumen of Rathke's pouch is dilated. The cyst was visible grossly.

The variation in the acinal epithelium from columnar to low cuboidal was considered to be the normal functional expressions of the gland. The remaining tissue, the heart and adrenal, failed to present significant changes.

The effect of a short term administration of carotene on vitamin A-deficient calves was investigated. The degree of the pathognomonic histopathological lesions, namely, squamous metaplasia of parotid gland interlobular and main ducts, was not influenced by the treatment (table 1). However, a statistically insignificant difference was noted in the form of better growth and feed consumption in favor of the carotene-treated calves. The blood plasma and the liver vitamin A values were slightly raised while the corresponding carotene values were increased considerably. Spinal fluid pressures were lower in the treated groups than in the controls, and in addition, the degree of diarrhea and muscular incoördination decreased.

In a similar experimental treatment, ascorbic acid and vitamin A were employed (table 1). The interrelationship between ascorbic acid and vitamin A in the deficient bovine has been claimed to be expressed by a lowered blood plasma level as well as a lowered urinary exeretion of ascorbic acid.[2,39] In the present study, ascorbic acid treatment was without any beneficial effect on depleted animals save to increase the ascorbic acid blood plasma level. However, administration of vitamin A for two weeks resulted in the complete disappearance of diarrhea and incoördination, and significant improvement in the parotid gland lesions. All of the interlobular ducts and half of the main ducts had returned to normal in the 6 treated calves. In the 3 animals which had not returned to normal completely, the pathognomic squamous metaplastic epithelium was being replaced by transitional epithelium, which suggested that the manner of reversal was from the pathological squamous epithelium to transitional epithelium to the normal pseudocolumnar type. There was a definite drop in the cerebrospinal fluid pressure. This fact would seem to indicate that increased spinal fluid pressure for a limited time caused little or no stenosis of the optic foramen. It did not seem plausible that a bony structure could revert to a normal state in such a brief period. The 4 animals which had been treated with vitamin A for four weeks instead of two weeks had completely recovered, according to chemical and pathological evidence except for the cerebrospinal fluid pressure (table 1). This was slightly higher than normal.

SUMMARY

Fifty-four dairy calves were raised on a diet of limited whole milk, dry calf starter, and hay until 105 days old, except for 5 which were raised until 63 days old. At that time all animals were placed on a diet deficient in vitamin A. Six of these calves were fed 107,143 U.S.P. units of vitamin A daily and served as controls. Twenty were depleted to 4 μg. of vitamin A per 100 cc. or less of blood plasma and slaughtered after two weeks at that level, while 4 were killed after seven weeks at that level. Of 8 depleted calves, 4 were given carotene orally and 4 intravenously for six days and slaughtered after fourteen days. Six calves were depleted and given ascorbic acid daily for fourteen days and slaughtered, 6 were treated the same but given vitamin A daily for fourteen days, 4 were given vitamin A daily for a 28-day period after depletion.

Observations consisted of ascertaining clinical symptoms, spinal fluid pressure, assays of carotene, and vitamin A levels in liver and blood plasma, and histopathological changes.

Diarrhea and incoördination were consistent aspects of vitamin A deficiency in calves. Spinal fluid pressure rose in the depleted animals. Histological examination revealed squamous metaplasia of interlobular and main ducts of the parotid gland in all but 1 of the deficient animals. Because of the frequency of this lesion, it was considered pathognomonic. Focal necrotic hepatitis was less frequent but appeared only in deficient animals. Changes in other organs such as the adrenal gland, intestinal tract, kidney, testis, and thyroid gland did not occur in specific relation to hypovitaminosis A. Only one pituitary gland was cystic and this in a deficient animal.

Carotene failed to reverse the pathognomonic lesions of the parotid gland, although it did ameliorate diarrhea and incoordination. Ascorbic acid failed to produce any improvement. Vitamin A feeding for two weeks induced partial reversal of the

squamous metaplasia, and for four weeks, complete recovery. Spinal fluid pressures were markedly reduced and symptoms disappeared when vitamin A was fed.

References

[1] Bassett, C. F., Harris, L. E., Smith, S. E., and Toeman, E. D.: Urinary Calculi Associated with Vitamin A Deficiency in the Fox. Cornell Vet., *36*, (1946): 5-16.

[2] Boyer, P. D., Phillips, P. H., Lundquist, N. S., Jensen, C. W., and Rupel, I. W.: Vitamin A and Carotene Requirements for the Maintenance of Adequate Blood Plasma Vitamin A in the Dairy Calf. J. Dai. Sci., *25*, (1942): 433-440.

[3] Bratton, R. W., Salisbury, G. W., Tanabe, T., Branton, C., Mercier, L., and Loosli, J. K.: Breeding Behavior, Spermatogenesis, and Semen Production of Mature Dairy Bulls Fed Rations Low in Carotene. J. Dai. Sci., *31*, (1948): 779-791.

[4] Coplan, Helen M., and Sampson, M. M.: The Effects of Deficiency of Iodine and Vitamin A on the Thyroid Gland of the Albino Rat. J. Nutr., *9*, (1935): 469-483.

[5] Creech, G. T., and Seibold, H. R.: Histopathology of Anasarcous Lesions Observed in Natural Cases of Vitamin A Deficiency in Cattle. Am. J. Vet. Res., *4*, (1943): 353-359.

[6] Davies, A. W.: The Colorimetric Determination of Vitamin A by Alkali Digestion. Biochem. J., *44*, (1949): 264-267.

[7] DeRuyter, T. H.: Acta brev. Neerland, *4*, (1934): 122. Quoted by Lipsett and Winzler.[28]

[8] deSchweinitz, G. E., and DeLong, P.: Blindness and Papilledema in Guernsey Calves. Arch. Ophth., *11*, (1934): 194.

[9] Eaton, H. D., Helmboldt, C. F., Jungherr, E. L., Carpenter, C. A., and Moore, L. A.: Effect of Vitamin A Depletion on Liveweight, Plasma, and Liver Levels of Vitamin A and Microanatomy in Young Dairy Calves. J. Dai. Sci., *34*, (1951): 386-395.

[10] Eaton, H. D., Matterson, L. D., Decker, Lois, Helmboldt, C. F., and Jungherr, E. L.: Intravenous and Oral Administration of an Aqueous Suspension of Carotene to Calves Depleted of Their Vitamin A Stores. J. Dai. Sci., *34*, (1951): 1073-1080.

[11] Erb, R. E., Andrews, F. N., Hauge, S. M., and King, W. A.: Observations on Vitamin A Deficiency in Young Dairy Bulls. J. Dai. Sci., *30*, (1947): 687-702.

[12] Follis, R. H.: The Pathology of Nutritional Disease. C. C. Thomas, Springfield, Ill., 1948.

[13] Ham, A. W.: Histology. J. B. Lipincott Co., Montreal, (1950): 531-540.

[14] Hansel, W., McEntee, K., and Olafson, P.: The Effects of Two Causative Agents of Experimental Hyperkeratosis on Vitamin A Metabolism. Cornell Vet., *41*, (1951): 367-376.

[15] Hart, G. H., and Guilbert, H. R.: Vitamin A Deficiency as Related to Reproduction in Range Cattle. California Agric. Exp. Sta. Bull., *560*, (1933): 3-30.

[16] Hart, G. H., and Guilbert, H. R.: Symptomatology of Vitamin A Deficiency in Domestic Animals. J.A.V.M.A., *44*, (1937): 193-200.

[17] Heimer, Caryl B., Maslow, H. L., and Sobel, A. E.: Influence of Thyroid on Utilization of Vitamin A. J. Nutr., *38*, (1949): 345-352.

[18] Hodgson, R. E., Hall, S. R., Sweetman, W. J., Wiseman, N. G., and Converse, H. T.: The Effect of Vitamin A Deficiency on Reproduction in Dairy Bulls. J. Dai. Sci., *29*, (1946): 669-689.

[19] House, E. L.: The Development of the Hypophysis of the Ox. Am. J. Anat., *73*, (1943): 1-25.

[20] Jungherr, E. L.: Nasal Histopathology and Liver Storage in Subtotal Vitamin A Deficiency in Chickens. Storrs (Conn.) Agric. Exper. Sta. Bull., *250*, 1943.

[21] Jungherr, E. L., Helmboldt, C. F., and Eaton, H. D.: Experimental Vitamin A Deficiency in Young Dairy Cattle. (Abstr.) J. Anim. Sci., *8*, (1949): 641.

[22] Jungherr, E. L., Helmboldt, C. F., and Eaton, H. D.: Parotid Gland Lesions in Experimental Bovine Vitamin A Deficiency. J. Dai. Sci., *33*, (1950): 666-675.

[23] Keener, H. A., Bechdel, S. I., Guerrant, W. B., and Thorp, W. T. S.: Carotene in Calf Nutrition. J. Dai. Sci., *25*, (1942): 571-583.

[24] Kimble, M. S.: The Photoelectric Determination of Vitamin A and Carotene in Human Plasma. J. Lab. Clin. Med., *24*, (1939): 1055-1068.

[25] Langham, Robert F.: A Clinical and Pathological Study of Vitamin A Deficiency in Calves. Doctoral Thesis, Michigan State College (1950) and Biol. Abstr., No. 6, 17919, *25*, 1951.

[26] Langham, R. F., Sholl, L. B., and Hallman, E. T.: The Pathology of the Bovine Kidney in Vitamin A Deficiency. Am. J. Vet. Res., *2*, (1941): 319-322.

[27] Lindley, C. E., Brugman, H. H., Cunha, T. J., and Warwick, E. J.: The Effect of Vitamin A Deficiency on Semen Quality and the Effect of Testosterone and Pregnant Mare Serum on Vitamin A-Deficient Rams. J. Anim. Sci., *8*, (1949): 590-602.

[28] Lipsett, M. B., and Winzler, R. J.: Effects of Vitamin A Deficiency on Thyroid Function Studied with Radioactive Iodine. Endocrinology, *41*, (1947): 494-500.

[29] Loosli, J. K., Huffman, C. F., Peterson, W. E., and Phillips, P. H.: Recommended Nutrient Allowances for Dairy Cattle. III. National Research Council, Washington, 1950.

[30] Madsen, L. L., and Earle, I. P.: Some Observations on Beef Cattle Affected with Generalized Edema or Anasarca Due to Vitamin A Deficiency. J. Nutr., *34*, (1947): 603-619.

[31] Madsen, L. L., Hall, S. R., and Converse, H. T.: Cystic Pituitary in Young Cattle with Vitamin A Deficiency. J. Nutr., *24*, (1942): 15-24.

[32] Mayer, Jean, and Truant, A. P.: Effects of Administration of Testosterone on Vitamin A-Deficient Rats. Proc. Soc. Exptl. Biol. Med., *72*, (1949): 436-438.

[33] Mayer, Jean, and Goddard, J. W.: Effects of Administration of Gonadotropic Hormone on Vitamin A-Deficient Rats. Proc. Soc. Exptl. Biol. Med., *76*, (1951): 149-151.

[34] McEntee, K.: Personal Communication. New York State Veterinary College, 1951.

[35] Moore, L. A.: Some Ocular Changes and Deficiency Manifestations in Mature Cows Fed a Ration Deficient in Vitamin A. J. Dai. Sci., *24*, (1941): 893-902.

[36] Moore, L. A., Huffman, C. F., and Duncan, C. W.: Blindness in Cattle Associated with a Constriction of the Optic Nerve and Probable of Nutritional Origin. J. Nutr., *9*, (1935): 533.

[37] Moore, L. A., and Hallman, E. T.: Production of White Spotted Kidneys in Calves. J. Dai. Sci., *19*, (1936): 434.

[38] Moore, L. A., and Sykes, J. F.: Terminal Cerebrospinal Fluid Pressure Values in Vitamin A Deficiency. Am. J. Physiol., *134*, (1941): 436-439.

[39] Moore, L. A., and Cotter, J. W.: The Relationship Between a Low Carotene Intake and Urinary Excretion of Ascorbic Acid in Dairy Cattle. J. Dai. Sci., *28*, (1945): 496-506.

[40] Osborn, T. B., Mendel, L. B., Terry, E. L.: The Incidence of Phosphatic Urinary Calculi in Rats Fed on Experimental Rations. J. Am. M. A., *69*, (1917): 32-33.

[41] Remington, R. E., Harris, P. L., and Smith, C. L.: Relationship Between Vitamin A and Iodine Metabolism in the Rat. J. Nutr., *24*, (1943): 597-605.

[42] Ritzman, E. G., Coloros, N. F., Keener, H. A., and Teeri ,A. E.: Influence of Vitamin A on the Utilization of Energy and Protein by Calves. Tech. Bull. *88*, New Hampshire Agric. Exper. Sta., (1945): 1-27.

[43] Roe, J. H., and Keuther, C. A.: The Determination of Ascorbic Acid in Whole Blood and Urine Through 2,4-Dinitrophenylhydrazine Derivative of Dehydroascorbic Acid. J. Biol. Chem., *147*, (1943): 399-408.

[44] Sampson, Myra M., and Korenchevshy, V.: The Influence of Vitamin A Deficiency on Male Rats in Paired Feeding Experiments. Biochem. J., *26*, (1932): 1322-1339.

[45] Schmidt, H.: Vitamin A Deficiencies in Ruminants. Am. J. Vet. Res., *2*, (1941): 373-389.

[46] Seifried, O.: Studies on A-avitaminosis in Chickens. J. Exptl. Med., *58*, (1930): 519-537.

[47] Sure, B.: Influence of Avitaminosis on Weights of Endocrine Glands. Endocrinology, *23*, (1938): 575-580.

[48] Sutton, T. S., and Brief, B. J.: Anatomical and Physiological Changes in the Pituitary Glands of Vitamin A-Deficient Rats. Ohio Agric. Exper. Sta. Bull. *610*, 1939.

[49] Sutton, T. S., Krause, W. E., and Hansard, L. S.: The Effect of Vitamin A Deficiency on the Young Male Bovine. J. Dai. Sci., *23*, (1940): 574.

[50] Sykes, J. F., and Moore, L. A.: The Normal Cerebrospinal Fluid Pressure and a Method for Its Determination in Cattle. Am. J. Vet. Res., *3*, (1942): 364-367.

[51] Thorp, W. T. S., Keener, H. A., Bechdel, S. T., and Guerrant, N. B.: Observations on the Pathology of Dairy Calves on Low Vitamin A Diets. Am. J. Vet. Res., *3*, (1942): 27-31.

[52] Trautmann, A., and Fiebiger, J.: Histologie und vergleichende mikroskopische Anatomie der Haussäugetiere. 9th ed. Paul Parey, Germany, (1949): 125-127.

[53] Votila, U.: Virchows Arch. f. path. Anat., *301*, (1938): 535. Quoted by Lipsett and Winzler.[25]

[54] Ward, R. E., Bechdel, S. I., and Guerrant, N. B.: Carotene and Vitamin A in the Nutrition of Growing Dairy Cattle. J. Dai. Sci., *23*, (1940): 115-124.

[55] Wetzel, J. O., and Moore, L. A.: Blindness in Cattle due to Papilledema. Am. J. Ophth., *23*, (1940): 499-513.

[56] Wilson, J. G., and Warkang, J.: Epithelial Keratinization as Evidence of Fetal Vitamin A Deficiency. Proc. Soc. Exptl. Biol. Med., *64*, (1947): 419-422.

[57] Wolbach, S. B., and Howe, P. R.: Tissue Changes Following Deprivation of Fat-Soluble A Vitamin. J. Exptl. Med., *42*, (1925): 753-777.

[58] Wolbach, S. B., and Bessey, O. A.: Relative Overgrowth of the Central Nervous System in Vitamin A Deficiency in Young Rats. Science, *91*, (1940): 599-600.

[59] Wolbach, S. B., and Bessey, O. A.: Tissue Changes in Vitamin Deficiencies. Physiol. Rev., *22*, (1942): 233-289.

Effect of Certain Drugs Alone and In Combination on Micrococci Isolated from the Bovine Udder

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ONE OF THE diseases of dairy cattle causing a tremendous annual economic loss is mastitis.[1, 2] Martinez [3] states that this disease is found widely prevalent in all countries of the world. Studies in Tennessee [4] indicate an average of 45 to 50 per cent of the cows in most herds carry detectable numbers of infectious organisms which are potentially pathogenic. An average of 73 per cent of animals in that area were shedding the infectious organisms in the milk during the entire year.

The immediate problem of the veterinarian and dairy cattle owner, when handling mastitis, is a method of treating the acute or chronic forms. The object is to return the dairy cow to normalcy and production in the milking line. Both acute local mastitis, with limitation of the pathological changes and bacterial infection to the udder, and acute systemic mastitis, which is accompanied by systemic symptomatology of existing toxemia and bacteremia, are of importance.

Animals with acute staphylococcal mastitis present the following symptoms.[5] There is marked depression, loss of appetite, decreased milk yield, generally high temperature, and the affected quarter is hot, tense, and painful. The secretion is scanty, odorless, and resembles clear serum. As the quarters clear, the secretion becomes thin, watery, and of a dirty white color before a normal milk is again produced.

Introduction of pathogenic microörganisms after predisposing factors, such as teat or udder injuries resulting from improper stabling, poor milking techniques with frequent and chronic irritation of the lining epithelium, enables the pathogens to set up infection with ensuing pathological changes. There is active congestion with destruction of the glandular epithelium. The lumina of the alveoli are filled with coagulated masses of exudate and clotted milk. Many of the alveoli contain desquamated cells and polymorphonuclear leukocytes. The congestion is accompanied by lymphocytic cellular infiltration into the area. Interlobular edema develops and the congested area becomes hot and painful.

Should the pathogenic microörganisms still be present after recovery from the externally inflicted damage to the udder, chronic productive mastitis follows. There is an increase in the interlobular connective tissue. This chronic mastitis may progress to the ultimate disappearance of most of the alveoli or functional glandular tissue. Such replacement is readily detected by palpation and is described by the dairyman as a meaty udder.

Murphy [6] suggests that the introduction of potentially pathogenic microörganisms does not mean that infection will naturally follow. He suggests the term "inhabitation" for the presence of organisms until some local condition develops to allow the microbial infective process to begin. From the standpoint of the udder, the invasion and infection phases must be largely passive in that the invading microörganisms are merely meeting and finding themselves capable or incapable of surmounting conditions already present.

The micrococci are ubiquitous, especially in animal quarters, and staphylococcal abscesses are common in both soft tissues and bone of cattle, horses, and other animals.

The resistant property of the staphylococci to drying is a predisposing factor to the high incidence of mastitis. Such resistant organisms serve as a potential reservoir at all times in dairy cattle. Exposure to a temperature of 60 C. for thirty to sixty minutes is often necessary to kill.

McEwen and Cooper [7] conclude that immunization, while presenting problems of scientific and academic interest, is unlikely to yield results that may be used successfully in the field. Instead, they proposed that chemotherapy holds out the best chance of mitigating the losses sustained by mastitis. They suggested further efforts be made to obtain better chemotherapeutic agents for use under dairy farm conditions.

From a thesis submitted to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of doctor of philosophy.

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The latter part of the work was completed at the Fort Dodge Laboratories, Fort Dodge, Iowa, and thanks are extended to those who have provided the facilities, equipment, and drugs to continue this part of the work.

Smith *et al.*[8] suggests that the initial infectivity of staphylococci for a given species depends largely upon their ability to clot plasma of that species, the role of the coagulase being to inhibit the phagocytosis of the cocci. The local inflammatory reaction which follows tissue invasion serves to wall off the infected area. There is polymorphonuclear leukocytic infiltration to protect against further spread and this is followed by the arrival of the macrophages to further control the infection.

Jungherr[9] confirmed the above with the suggestion that histopathological specimens show the causative organisms within the tissues in chronic staphylococcal infections, whereas, in chronic streptococcal infection, the organisms appeared in the lumina of the milk ducts only. This is proposed as one reason why staphylococcal infections, once well established, do not respond as well as streptococcal infections to intramammary infusions.

Because of the tremendous annual economic losses due to mastitis in dairy cattle, investigations into medicinal agents which might be of benefit have been carried on for years.

In a report released by the committee of animal health, National Research Council,[10] the value and limitations of intramammary therapy were discussed with summaries of results obtained in controlled investigations on each of the chemotherapeutic and antibiotic agents available for treating bovine mastitis. It was pointed out that most investigators were agreed that 50 to 90 per cent of udder infections due to *Streptococcus agalactiae* are curable.

TABLE 1—Basic Information on the Pathogenic Micrococcus Pyogenes var. Aureus Strains Included in this Report

Culture (No.)	Date isolated	Comments
H263-2	1-8-49	Chronic mastitis; no history of treatment
682-4	7-25-49	Mastitis; no treatment prior to isolation
13-4	3-18-50	Chronic mastitis; no history of treatment
12-4	3-18-50	Chronic mastitis; no history of treatment
H85-2	3-15-49	Mastitis; no history of treatment
E570-4	4-29-49	Mastitis; cow died with acute mastitis
847-4	6-20-49	Acute mastitis; 900,000 units penicillin repeated 4 times; sulfonamide i.v. and p.o.
H129-1	3-15-49	Mastitis; no history of treatment
831-4	3-11-49	Chronic mastitis; 40,000 units penicillin i.m.
643-4	4-9-49	Chronic mastitis; no treatment

i.v.—intravenous; p.o.—per os; i.m.—intramuscular.

Also included was the information that staphylococci were found in the milk from as high as 30 to 40 per cent of the cows. Frequently, the mastitis produced by these organisms was characterized by swelling, pain, scanty, abnormal secretion associated with generalized toxemia which may be even the cause of death. The majority of reports show such infection to be exceedingly difficult to eliminate from the udder. Even penicillin was not as effective against staphylococcal as against streptococcal infection.

TABLE 2—Method for Testing Antibiotic Activity Against the Micrococcal Strains

Tube	Broth (ml.)	Antibiotic in broth (ml.)	Inoculum (ml.)	Total volume (ml.)	Units or micrograms (ml.)
1	0.0	0.5	0.5	1.0	5.0
2	0.3	0.2	0.5	1.0	2.0
3	0.4	0.1	0.5	1.0	1.0
4	0.0	0.5	0.5	1.0	0.5
5	0.3	0.2	0.5	1.0	0.2
6	0.4	0.1	0.5	1.0	0.1
7	0.0	0.5	0.5	1.0	0.05
8	0.3	0.2	0.5	1.0	0.02
9	0.4	0.1	0.5	1.0	0.01
10	0.5	0.0	0.5	1.0	0.0

The prevalency of staphylococci in mastitis which now is not successfully treated, a study of their pathogenicity, their susceptibility to various antibiotics, combinations of antibiotics, and combinations of antibiotics and sulfonamides serve as topics which might be profitably considered as a subject of further research. I will report herein the data obtained, materials and methods used, with a discussion of the results.

METHODS AND MATERIALS

The following strains of staphylococci were isolated in pure culture and their growth habits on tryptose agar slants and in tryptose phosphate broth mediums indicated they would be suitable to use in the study of the inhibitory effects of the antibiotics or antibiotic combinations on them. A method similar to that described by Hartman[11] was used to isolate the strains.

The udder and teats were washed with an antiseptic chlorinated solution and dried. The tips of the teats were wiped with a cotton pledget saturated with 70 per cent alcohol. Four to five streams of milk were squirted into a strip cup and then 15 ml. of milk was collected into a sterile tube and capped. This carefully obtained sample was used to plate out the organisms and obtain a pure culture.

The information included in table 1 indicates the

culture number of the strains used in this study. The cow's quarters were numbered as the terminal digit in the strain number, as follows: 1 = RF = right fore, 2 = RR = right rear, 3 = LF = left fore, 4 = LR = left rear.

A preliminary screening was done on each culture to determine whether adequate visible turbidity would develop in twenty-four hours to make reading of the end point more accurate. Twenty-four hour tryptose phosphate broth cultures were used to inoculate broth mediums which were to serve as the inoculum for inhibitory studies. At the time of the test, a loopful of the 24-hour culture of each strain was transferred to a tube containing 10 ml. of sterile broth.

From the second tube, or tube B, containing 1 loopful of culture from tube A, dispersed in 10 ml. of broth, 0.5-ml. samples were taken as the culture inoculum for trial against the antibiotics, sulfonamides, or combinations of these drugs. The outline of the procedure as presented in table 2 follows the technique as utilized by Rammelkamp and Maxon.[12]

The antibiotics were dissolved in broth so that 10 units or micrograms were present per milliliter in the medium used in the first three tubes. A volume of 0.5 ml. thus furnished 5 units or micrograms for tube 1, 0.2 ml. supplied 2 units or micrograms for tube 2, and 0.1 ml. gave 1 unit or microgram in tube 3. Another preparation of the antibiotic in the tryptose phosphate broth, to provide a level of 1 unit or microgram, was used in correct amounts for tubes 4, 5, and 6. In a similar way, 0.1 units or micrograms were dissolved in such medium for supplying the proper amounts for tubes 7, 8, and 9. Tube 10 served as a control.

TABLE 3—Inhibition of Strains of Staphylococci by Single Antibiotics

Strain	Minimum concentrations per milliliter preventing visible growth			
	Penicillin (units)	Streptomycin (μg.)	Aureomycin (μg.)	Terramycin (μg.)
H263-2	5.0	5.0	2.0	0.5
682-4	0.2	0.5	0.5	0.5
13-4	0.5	5.0	1.0	2.0
12-4	0.5	5.0	2.0	2.0
H85-2	0.2	0.1	1.0	1.0
E570-4	0.2	0.2	0.5	1.0
847-4	0.1	0.1	0.5	0.5
H129-1	0.2	0.5	2.0	2.0
831-4	0.2	0.5	1.0	1.0
643-4	0.2	1.0	0.5	0.2

The strains of staphylococci isolated and used in this project possessed the general characteristics of the group *Micrococcus pyogenes*, var. *aureus*. They were gram positive and grew well in the artificial mediums tried. Adequate growth in tryptose phosphate broth was the basis of selection of such mediums for this work. The growth on solid mediums was characteristic for each of the strains.

The data presented in table 3 indicates the minimum level of single antibiotics required to inhibit visible growth of the several strains for twenty-four hours. These results are based on three trials for each strain with each antibiotic at the several levels. Two of the three of these trials at each level were identical and this level was the one reported herein.

Sulfisoxazole, (Gantrisin, Hoffman - La-Roche) has been reported by numerous investigators to be effective in controlling gram positive and negative coccal infections under experimental and clinical conditions. The original plan of this project was to include one of the sulfonamides in combination with the antibiotics to determine the possible synergistic chemotherapeutic activity of such combinations. Price *et al.*[13] described a similar plan for antibiotic combinations with sulfamerazine, sulfathiazole, and sulfadiazine.

Prior to using a combination of sulfisoxazole and the antibiotics, it was desirable to determine the possible inhibitory activity of this drug in tryptose phosphate broth against the strains of micrococci being studied. The results reported in table 4 indicate that most strains grew at a level of 500 μg. per milliliter of sulfisoxazole.

When, however, this sulfonamide was combined with certain antibiotics, it was found that the concentration of the latter, necessary to inhibit visible growth, was reduced. Thus, the data presented in tables 5 and 6 indicate potentiation of antibiotic activity in those instances marked with an asterisk. All strains grew readily at a

TABLE 4—Influence of Several Concentrations of Sulfisoxazole on the Growth of Staphylococcal Strains in Vitro

Strain	Concentration of sulfisoxazole in each tube expressed in micrograms per milliliter				
	50	100	150	250	500
H263-2	+	+	+	+	+
682-4	+	+	+	+	+
13-4	+	+	+	+	+
12-4	+	+	+	+	+
H85-2	+	+	+	−	−
E570-4	+	+	+	−	−
847-4	+	+	+	+	−
H129-1	+	+	+	+	+
831-4	+	+	+	+	+
643-4	+	+	+	+	+

+ —visible growth after incubation for 24 hours at 37 C.
− —no growth after incubation for 24 hours at 37 C.

level of 50 μg. per milliliter of sulfisoxazole; therefore, the increased antibacterial action was a result of the combined action of the drugs.

Another point of interest concerning the possible combination of sulfonamides and antibiotics would be the trial of certain strains of the staphylococci with several levels of sulfisoxazole. The required concentration of the antibiotics needed to prevent the visible growth was found to be the same at levels of 50, 100, and 250 μg. per milliliter of sulfisoxazole.

TABLE 5—Influence of Sulfisoxazole on the Inhibitory Activity of Certain Antibiotics in Comparison to Action of Single Antibiotics

Strain	Concentrations in units or micrograms preventing visible growth				
	P (units)	SS	P + SS (units*)	S (μg.)	S + SS (μg.)
H263-2	5.0	+	0.02	5.0	5.0
682-4	0.2	+	0.02	0.5	0.1 *
13-4	0.5	+	0.02	5.0	5.0
12-4	0.5	+	0.02	5.0	2.0 *
H85-2	0.2	+	0.01	0.1	5.0
E570-4	0.2	+	0.005	0.2	2.0
847-4	0.1	+	0.02	0.1	2.0
H129-1	0.2	+	0.02	0.5	2.0
831-4	0.2	+	0.02	0.5	1.0
643-4	0.2	+	0.02	1.0	2.0

P = Penicillin; SS = sulfisoxazole (+) growth occurred in all strains at a level of 50 μg. per milliliter; S = streptomycin.
* = Potentiation of antibiotic activity.

Previous investigators have presented results indicating that certain combinations of antibiotics may demonstrate inhibitory activity superior to that of single antibiotics. Foley *et al.*[14] reported that penicillin alone had not been too effective in controlling mastitis due to staphylococci. Two explanations were offered. Some strains of staphylococci produce penicillinase and thus a higher level of penicillin must be maintained to achieve bactericidal action. Also, strains that were originally relatively susceptible may develop into resistant types. Scott,[15] too, noted the marked parallelism between the susceptibility of strains of coagulase positive staphylococci to penicillin and aureomycin.

It was deemed important to also investigate the effect of combinations of antibiotics for possible therapeutic combinations to be used in treating staphylococcal mastitis. Several plans could be followed for dilutions of the antibiotics for observing the synergistic, potentiation, or decreased effectiveness by mixtures of the antibiotics. Such combinations might have three possible effects; the combined effect might be additive, synergistic, or, finally, the mixture could show a lesser effect than the sum of the effects of the active constituents.

This phase of investigation was planned to include dilutions of the antibiotics so that each tube would contain 0.5 ml. of a 24-hour broth culture, a combination of antibiotics at a level of 5 units of penicillin plus 5μg. of the other antibiotics per milliliter in the first tube, 2 units of penicillin plus 2 μg. of the other antibiotic in the second tube and thus by further steps of dilution to the control tube with only broth and culture. In those not including penicillin, two other antibiotics and dilutions of these amounts, as described above, are shown in table 7. Sufficient broth was added to each tube to give a total volume of 1 ml. of culture, antibiotic in broth, and plain tryptose phosphate broth.

The necessary level of the two combined antibiotics to inhibit visible growth is presented in table 8. For example, visible growth of strain H263-2 was inhibited by a concentration of 0.02 units of penicillin and 0.02 μg. of streptomycin.

The *in vitro* results obtained thus far on these strains of staphylococci confirmed their sensitivity to penicillin, streptomycin, aureomycin, and terramycin. Higher levels in antibiotics were necessary to inhibit visible growth in tryptose phosphate broth for some strains than for others. Strain H263-2 represented one requiring higher levels than strain 682-4.

The sulfonamide, sulfisoxazole, alone in broth cultures with the ten strains, was not effective in inhibiting growth at a level of 50 or 100 μg. per milliliter. However, when it was added at a level of 50 μg. per milliliter with penicillin, aureomycin, or terramycin, there was enhancement of the antibacterial activity of the antibiotic. This

TABLE 6—Influence of Sulfisoxazole on the Inhibitory Activity of Certain Antibiotics in Comparison to Action of Single Antibiotics

Strain	Concentrations in units or micrograms preventing visible growth				
	A (μg.)	SS	A + SS (μg.*)	T (μg.)	T + SS (μg.)
H263-2	2.0	+	0.05	0.5	0.2 *
682-4	0.5	+	0.05	0.5	0.1 *
13-4	1.0	+	0.05	2.0	0.1 *
12-4	2.0	+	0.05	2.0	1.0 *
H85-2	1.0	+	0.05	1.0	1.0
E-570-4	0.5	+	0.01	1.0	0.5 *
847-4	0.5	+	0.01	0.5	1.0
H129-1	2.0	+	0.02	2.0	0.5 *
831-4	1.0	+	0.05	1.0	1.0
643-4	0.5	+	0.01	0.2	1.0

A = Aureomycin; SS = sulfisoxazole (+) growth occurred in all strains at a level of 50 μg. per milliliter; T = terramycin.
* = Potentiation of antibiotic activity.

synergistic action with sulfisoxazole and penicillin was evident in all ten strains; in all ten strains with aureomycin and sulfisoxazole, and with six out of ten strains

with terramycin and sulfisoxazole. An equal or higher concentration of streptomycin was required for inhibition of visible growth in eight out of ten strains when sulfisoxazole was added than when streptomycin was used alone.

Combinations of the antibiotics penicillin and streptomycin, terramycin and streptomycin, terramycin and aureomycin uniformly reduced the levels of antibiotic required to inhibit visible growth. Combinations of penicillin and terramycin and penicillin and aureomycin showed only slight or no increase in effectiveness on the several strains.

TABLE 7--Procedure for Dilution of Antibiotics for Single or Combined Action

Volume of broth containing antibiotic(s) (ml.)	Amount of antibiotic present in units or micrograms per milliliter			
	Penicillin (units)	Streptomycin (μg.)	Aureomycin (μg.)	Terramycin (μg.)
0.5	5.0	5.0	5.0	5.0
0.2	2.0	2.0	2.0	2.0
0.1	1.0	1.0	1.0	1.0
0.5	0.5	0.5	0.5	0.5
0.2	0.2	0.2	0.2	0.2
0.1	0.1	0.1	0.1	0.1
0.5	0.05	0.05	0.05	0.05
0.2	0.02	0.02	0.02	0.02
0.1	0.01	0.01	0.01	0.01
0.0	Control	Control	Control	Control

DISCUSSION

Price *et al.*[16] indicated the intensive search being made to either discover new antibiotic agents or to determine antibiotic combinations which will provide more effective remedies. The ultimate purpose of such combinations is to insure a more rapid and effective eradication of the causative organisms. These workers demonstrated that the synergistic action in many of the beneficial *in vitro* results showed correlation with *in vivo* results. The importance of *in vivo* testing prior to introduction of a product for clinical usage was stressed.

The use of penicillin alone in treating bovine mastitis was reviewed by Jepsen.[17] He suggested that penicillin is active only against dividing bacteria and that it is necessary to maintain an active concentration in the udder as long as bacteria can divide. This was estimated to be five to six days. The duration could be maintained properly by giving 25,000 units of sodium penicillin in water per quarter for five to six doses at 24-hour intervals. Another form of therapy suggested was administration of two doses of 100,000 units per quarter at 72-hour intervals. He also reported local inactivation of penicillin in the inflamed quarter. Evidently, some penicillin was bound to certain constituents of the milk.

Spencer *et al.*[18] reviewed the use of antibacterial drugs for treating bovine streptococcic mastitis. They found penicillin as efficacious as aureomycin or a combination of sulfadiazine and penicillin. Jackson[19] reported that procaine penicillin in oil with aluminum monostearate, when given at a rate of 300,000 units, produced an effective antibacterial level for seventy-two hours at the beginning of lactation and for 192 hours during the middle of the lactation period.

Other workers[20-26] have investigated the action of the antibiotics singly and in com-

TABLE 8—Levels of Antibiotics in Combination Necessary to Inhibit the Staphylococcal Strains

Cultures	P	S	A	T	P + S	P + A	P + T	S + T	T + A
H263-2	5.0	5.0	2.0	0.5	0.02 *	1.0 *	0.2 *	0.02 *	0.05 *
682-4	0.2	0.5	0.5	0.5	0.05 *	1.0	2.0	0.02	0.05 *
13-4	0.5	5.0	1.0	2.0	0.05 *	1.0	1.0 *	0.02 *	0.01 *
12-4	0.5	5.0	2.0	2.0	0.02 *	1.0 *	1.0 *	0.02 *	0.01 *
H85-2	0.2	0.1	1.0	1.0	0.01 *	1.0	1.0	0.02 *	0.05 *
E570-4	0.2	0.2	0.5	1.0	0.02 *	1.0	2.0	0.01 *	0.05 *
847-4	0.1	0.1	0.5	0.5	0.02 *	1.0	1.0	0.01 *	0.01 *
H129-1	0.2	0.5	2.0	2.0	0.02 *	1.0 *	0.5 *	0.02 *	0.01 *
831-4	0.2	0.5	1.0	1.0	0.01 *	1.0	1.0	0.02 *	0.01 *
643-4	0.2	1.0	0.5	0.2	0.02 *	1.0	0.5	0.01 *	0.05 *

P—Penicillin, concentration of penicillin in units per milliliter causing inhibition of visible culture growth.

S—Streptomycin, concentration of streptomycin in micrograms per milliliter causing inhibition of visible culture growth.

A—Aureomycin, concentration of aureomycin in micrograms per milliliter causing inhibition of visible culture growth.

T—Terramycin, concentration of terramycin in micrograms per milliliter causing inhibition of visible culture growth.

* = Combinations showing increased antibacterial activity.

binations in controlling these infectious processes of animals, including man. Bigler [27] and others have reported on the relative effectiveness of sulfisoxazole against both gram-positive and gram-negative organisms. This relatively new sulfonamide has the advantages of being readily soluble and having a low toxicity.

It was the original purpose of this research to determine whether combinations of antibiotics or combinations of antibiotics and sulfonamides would show greater bacteriostatic or bactericidal activity *(in vitro)* than single antibiotics. As reported herein, the *in vitro* results were interpreted on the basis of inhibition of visible growth. More recently, Jackson *et al.*[28] described a turbidimetric method for determining the sensitivity of bacteria to antibiotics. As a part of the work, too, it was desired to determine whether these strains, as isolated from cows with clinical mastitis, were resistant to the penicillin and certain of the other antibiotics.

On examination of the results presented in table 3, it may be seen that the strains of staphylococci were sensitive to penicillin, streptomycin, aureomycin, and terramycin. Finland *et al.*[29] also reported on the general susceptibility of pathogenic staphylococci to antibiotics. From these same results, one may note the relatively greater amount of antibiotic needed to inhibit certain of the ten strains, with H263-2 being representative of the more resistant strains. On the other hand, strain 682-4 represents the types requiring a lower concentration of antibiotic for inhibition.

Prior to making a suggestion of additive or synergistic action, it became necessary to determine the effective inhibitory level of sulfisoxazole against the strains of staphylococci. Actually, it was found that only two of the strains were effectively inhibited at a level of 250 μg. per milliliter. A level of 500 μg. per milliliter did not inhibit the other strains, with the one exception of 847-4 and the two strains, H85-2 and E570-4, previously inhibited at a level of 250 μg. per milliliter.

However, the addition of only 50 μg. per milliliter of sulfisoxazole to penicillin, aureomycin, and terramycin resulted in marked improvement of antibacterial action.

This was thought to be of significance since this lower level would generally be present in the udder tissues and milk of dairy cows receiving effective oral therapy with such a sulfonamide.

As pointed out previously, other research workers have reported synergism or potentiation of inhibitory action with certain antibiotic combinations. The data presented in table 8 indicate certain combinations, penicillin and streptomycin, streptomycin and terramycin, as well as aureomycin and terramycin, uniformly showed potentiation above the results produced by these single antibiotics. However, penicillin and terramycin, and penicillin and aureomycin showed potentiation for strains H263-2, 13-4, 12-4, and H129-1 but more antibiotic was required for the other strains.

The exact reasons why certain combinations should be more effective than others are not known. Possibly, two antibiotics, which show similar antibacterial activity, act similarly on the metabolic cycle of the microörganism. For certain bacterial strains, this may result in potentiation of antibiotic activity while in other strains, if the antibiotics act at different points of the metabolic cycle, there may be still greater potentiation.

Chandler *et al.*[30] reported another interesting factor which may influence antibiotic activity that might be involved in these results. Resistant strains of staphylococci were found capable of producing penicillinase. However, this ability to produce penicillinase could be inhibited or lost by exposure to another antibiotic, aureomycin, and this loss was apparently irreversible.

Penicillin was shown earlier to act by preventing the concentration of glutamic acid within the staphylococci. Gale [31] suggested that penicillin-resistant staphylococci do not concentrate glutamic acid within the cells and no longer require the presence of preformed amino acids and nicotinamide for growth.

CONCLUSIONS

Ten strains of coagulase-positive staphylococci were isolated from dairy cows showing clinical symptoms of mastitis.

These strains were tested *in vitro* by the broth turbidity method for the possible antibacterial activity of single antibiotics, or combinations of antibiotics, and combinations of the antibiotics and the sulfonamide, sulfisoxazole.

These strains showed varying degrees of

sensitivity *in vitro* to penicillin, streptomycin, aureomycin and terramycin. Combinations of penicillin and streptomycin, terramycin and streptomycin, and terramycin and aureomycin showed potentiation of antibiotic activity over the single drugs. Combinations of penicillin and terramycin as well as penicillin and aureomycin in general did not show potentiation.

Addition of sulfisoxazole at a level of 50 μg. per milliliter showed a great increase in antibacterial effect against most of the ten strains when combined with penicillin, terramycin, and aureomycin. The addition of this sulfonamide along with streptomycin generally necessitated use of an increased level of streptomycin to prevent visible growth.

It is believed the superior *in vitro* results produced by the addition of the drug sulfisoxazole to the antibiotics penicillin, terramycin, and aureomycin as well as the synergistic improvement in inhibitory action of certain combinations of the antibiotics warrants the suggestion that clinical trials be made with these combinations for cases of bovine mastitis caused by sensitive staphylococcal strains.

References

[1] Brose, Cyrus P.: Pertinent Points in the Control of Mastitis in Dairy Cattle. Station Circular 108, August, 1950, The State College of Washington, Institution of Agricultural Science, Washington Agric. Exper. Sta.

[2] Bryan, C. S.: Some Practical Aspects of Milk Hygiene. Michigan Agric. Exper. Station Quart. Bull. *30*, (1948): 3, 251-257.

[3] Martinez, C. Ruiz: La Mamitis de la vaca, Ministerio de Agricultura y Cria, Tallares Craficos del Ministerio de Agricultura, Caracos, Venezuela, August, 1948.

[4] Swanson, Eric W., and Throop, B. T.: Mastitis Control Investigation. The University of Tennessee, 61st Ann. Rep., (1948): 68.

[5] Hulse, N. C.: The Treatment with Aureomycin of Acute Staphylococcal Mastitis. Vet. Rec. *63*, (1951): 439.

[6] Murphy, J. M.: The Genesis of Bovine Udder Infection and Mastitis. Am. J. Vet. Res. *8*, (1947): 29.

[7] McEwen, A. D., and Cooper, M. B.: Mastitis. Vet. Rec., *59*, (1947): 655.

[8] Smith, W., Hale, J. N., and Smith, M. N.: The Role of Coagulase in Staphylococcal Infections. Brit. J. Exptl. Path. *28*, (1947): 57-67.

[9] Jungherr, E. L., Heimboldt, C., and Plastridge, W. N.: Correlation of the Gross and Histopathology of Affected Quarters with Clinical and Bacteriological Evidence of Mastitis. Bulletin 271, Storrs Agric. Exper. Sta.

[10] The Intramammary Therapy of Bovine Mastitis, Rep. No. 5, Committee on Animal Health, National Research Council, J. A. V. M. A., *108*, (1946): 127-135.

[11] Hartman, A. Dean: Mastitis Investigations, Master's Thesis, Texas A. & M. College, August, 1941.

[12] Rammerlkamp, C. H., and Maxon, T.: Resistance of *Staphylococcus Aureus* to the Action of Penicillin. Proc. Soc. Exptl. Biol. and Med., *51*, (1942): 386-389.

[13] Price, C. W., Randall, W. A., Welch, H., and Chandler, V.: Studies of the Combined Action of Antibiotics and Sulfonamides. Am. J. Pub. Health, *39*, (1949): 340.

[14] Foley, E. J., and Byrne, J. V.: Observations on the Treatment of *Streptococcus Agalactiae* and *Staphylococcus Aureus* Udder Infections with Mixtures of Streptomycin and Penicillin. Cornell Vet., *39*, (1949): 243.

[15] Scott, E. G.: Aureomycin Sensitivity of Organisms Tested by the Bondi Disc Technique. Am. J. Clin. Path., *20*, (1950): 65.

[16] Price, C. W., Randall, W. A., Welch, H., and Chandler, V.: Studies of the Combined Action of Antibiotics and Sulfonamides. Am. J. Pub. Health, *39*, (1949): 340.

[17] Jepsen, A.: Penicillin Therapy of Mastitis. Nord. Vet. Med., *2*, (1950): 447-480.

[18] Spencer, G. R., Kraft, M. E., Simon, J., and Schenk, M.: Antibacterial Drugs in Bovine Mastitis. J.A.V.M.A., *117*, (1950): 414-418.

[19] Jackson, W. F., and Bryan, C. S.: Penicillin Milk Levels in Cows Following Intramammary Administration, Vet. Med., *45*, (1950): 395-399.

[20] Yow, E. M., Spink, W. W.: Advances in Antibiotic Therapy. Bull., Univ. of Minnesota Hospitals and Minnesota M. Founda., *20*, (1949): 218.

[21] Braude, A. I., and Spink, W. W.: The Action of Aureomycin and Other Chemotherapeutic Agents in Experimental Brucellosis. J. Immunol., *63*, (1950): 185.

[22] Bliss, E. A., Warth, P. T., Chandler, C. A.: The Susceptibility of Gram Positive Cocci, Gram Negative Bacilli, and Clostridia to Terramycin. Ann. New York Acad. Sci., *53*, (1950): 277.

[23] Gunnison, J. B., Jawetz, E., Coleman, J. R.: The Effect of Combinations of Antibiotics on Enterococci *in Vitro*. J. Lab. and Clin. Med., *36*, (1950): 900.

[24] Paine, T. F.: The Similarity on Action of Bacitracin and Penicillin on the Staphylococcus. J. Bact., *61*, (1951): 259.

[25] Armstrong, C. W. J., Larner, A. E.: The Effect of Combinations of Antibiotics on *Pseudomonas Aeruginosa* and on *Proteus Vulgaris in Vitro* and *in Vivo*. J. Lab. and Clin. Med., *37*, (1951): 584.

[26] Bliss, E. A., Warth, P. T., Chandler, C. A., and Long, P. H.: Studies of Terramycin, *in Vivo* and *in Vitro*. Bull., Johns Hopkins Hosp., *87*, (1950): 171.

[27] Bibler, J. A., and Thomas, O.: Clinical Observations on a New Sulfonamide (Gantrisin). Am. J. Dis. Child., *79*, (1950): 785.

[28] Jackson, G. G., and Finland, M.: Turbidimetric Method for Determining Sensitivity of Bacteria to Antibiotics. J. Lab. and Clin. Med., *38*, (1950): 289-307.

[29] Finland, M., Frank, Paul F., Wilcox, Claire: *In Vitro* Susceptibility of Pathogenic Staphylococci to Seven Antibiotics. Am. J. Clin. Path., *20*, (1950): 325.

[30] Chandler, C. A., Davidson, V. Z., Long, P. H., and Monner, J. J.: Studies on Resistance of Staphylococci to Penicillin; the Production of Penicillinase and Its Inhibition by the Action of Aureomycin. Bull. Johns Hopkins Hosp., *89*, (1951): 81-89.

[31] Gale, E. F., and Rodwell, A. W.: Amino Acid Metabolism of Penicillin Resistant Staphylococci. J. Bact., *55*, (1948): 161-167.

A Modification of the Middlebrook-Dubos Hemagglutination Test for Use in the Diagnosis of Johne's Disease

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ALTHOUGH the intradermal johnin test is widely used, there is a recognized need for a more sensitive test for use in herds where Johne's disease is particularly difficult to eradicate. Research workers have been constantly trying to improve the methods of testing for Johne's disease. The complement-fixation (c.f.) test using a lipoid antigen has given disappointing results in that reactions were obtained with serums from many apparently normal cattle.[1]

More recently, Sigurdsson *et al.*[2] recovered a complement-fixing antigen from the intestinal mucosa of sheep suffering from Johne's disease and found the c.f. test superior to the intradermal johnin test for testing sheep suspected of harboring Johne's disease. Hole[3] used a washed saline suspension of *Mycobacterium paratuberculosis* as an antigen in a modified c.f. test and reports that results up to the present suggest that the test, in tuberculosis-free animals, is a valuable aid to the confirmation of the disease in suspected cases.

Middlebrook and Dubos[4] described a hemagglutination test for the detection of antibodies in serums obtained from tubercular human beings and rabbits. The test was modified by Scott and Smith[5, 6] who substituted a commercial tuberculin for sensitizing the erythrocytes instead of the sensitizing extract prepared from tubercle bacilli used by Middlebrook and Dubos. The test was further modified by Fisher and Gregory[7] who used an extract of human tubercle bacilli and added the complement. They read the test as a hemolytic reaction. Using this procedure, they obtained positive reactions with serums obtained from cattle infected with Johne's disease as well as those infected with bovine tuberculosis. Fisher[8] showed that erythrocyte adsorbable material extracted from several species of Mycobacterium, including *M. paratuberculosis*, would react not only with homologous antiserums but also with heterologous Mycobacterium antiserums.

Sohier *et al.*[9] used a precipitated tuberculin prepared by the Pasteur Institute for sensitizing the erythrocytes which were used in testing serums from tubercular human beings and rabbits. This precipitated material was also used by Sohier *et al.*[10] for sensitizing erythrocytes used for testing serums from tuberculosis-infected cattle. Gernez-Rieux *et al.*[11] found that serums from paratuberculosis-infected cattle would react with either an extract of *M. tuberculosis* or tuberculin precipitate.

The following experiment was planned to determine if a hemagglutination test using johnin or a derivative of johnin in the preparation of the antigen would be of value in the diagnosis of Johne's disease in cattle.

PROCEDURE

Serum specimens were obtained from the following five groups of cattle: (1) animals artificially sensitized with *M. paratuberculosis* by intraperitoneal injection of 1 Gm. of killed bacilli suspended in 35 ml. of sterile mineral oil; (2) animals artificially sensitized to *M. tuberculosis* var. *bovis* by an injection of bacilli as described above (all animals in groups 1 and 2 were reactors to both intradermic johnin and tuberculin tests); (3) nonreactor cattle in a herd from which tuberculin reactors showing lesions had recently been removed; (4) animals in a herd with a history of Johne's disease; (5) cattle from three herds which had never experienced losses from Johne's disease or tuberculosis and were negative to both intradermic johnin and tuberculin tests; however, one of these herds was pastured adjacent to premises having cattle infected with Johne's disease.

Preparation of the Serum.—Ten milliliter of blood was drawn from each animal, allowed to clot at room temperature, and centrifuged. One milliliter of serum was removed from each specimen, added to 1 ml. of Eagle's saline,[8] and inactivated in a water bath at 65 C. for three minutes. To remove heterophile aggultinins, 0.4 ml. of washed packed sheep erythrocytes was added to each tube containing the serum-saline mixture and the tube was shaken immediately. After standing for thirty minutes, the tube was centrifuged at 2,500 r.p.m. for seven minutes, the serum drawn off, and the cells discarded.

Preparation of Erythrocytes.—Twenty-five milliliter of sheep's blood was drawn into an equal amount of modified Alsever's solution.[8] The mixture was centrifuged for ten minutes at 1,800 r.p.m., the supernatant fluid discarded, and the packed cells diluted with 6 volumes of Eagle's saline. This process was repeated three times, except that the mixture was centrifuged for twenty minutes at 2,000 r.p.m. on the last centrifugation, after which the supernatant was discarded, the packed erythrocytes were stored at 7 C., and used within forty-eight hours.

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Preparation of Antigen.—In preliminary experiments, johnin culture filtrate and intradermic johnin were used as sensitizing agents. Erythrocytes sensitized with these products gave inconsistent and unreliable results when mixed with 87 separate serums of 15 known positive animals. These products were discarded and a purified protein derivative (P.P.D.) [12] was used as the sensitizing agent. To prepare this product, cultures of *M. paratuberculosis* grown on Dorset and Henley's synthetic medium were selected at random at the time they would ordinarily be harvested and 2.0 ml. of 0.5 molar solution of Na_2HPO4 were added to each 100-ml. quantity. After seven day's additional incubation, the cultures were placed in flowing steam for three hours, filtered, the bacilli discarded, 1/10 volume of 40 per cent trichloroacetic acid added to the filtrate, and the mixture held at 7 C. for twelve hours. At this time, the supernatant was siphoned off and the precipitate washed with 1 per cent trichloroacetic acid, centrifuged, and the supernatant discarded. The precipitate was washed and centrifuged three times with 10 volumes of anhydrous acetone followed by three more washings, each with 10 volumes of ether. The P.P.D. was dried by vacuum desiccation.

To reconstitute, 10 mg. of the desiccated material was dissolved in 6 ml. of saline. One-tenth milliliter of the washed packed erythrocytes was sensitized by adding the P.P.D. solution and incubating for two hours at 37 C., during which time the suspension was shaken every fifteen minutes. The cells were recovered after sensitization by centrifugation at 1,000 r.p.m. for six minutes, resuspended in 6 ml. of saline, and centrifuged again. The washing process was repeated three times and the cells were finally suspended in 50 ml. of saline. This 0.2 per cent suspension of sensitized cells constituted the antigen used in the hemagglutination test. The antigen was stored at 7 C. until used within forty-eight hours.

The Hemagglutination Test.—Seven standard Wasserman tubes were set up for each serum. Four-tenths milliliter of saline was added to all tubes except the last. Four-tenths milliliter of adsorbed serum-saline mixture was added to the first and last tubes. Twofold serial dilutions were made from the first tube through the sixth tube. Four-tenths milliliter of antigen was added to each serum-saline mixture except the last. Four-tenths milliliter of a normal 0.2 per cent cell suspension was added to the seventh tube as a control for incomplete adsorption of heterophile agglutinins. As a control for spontaneous clumping, 0.4 ml. of the antigen was added to 0.4 ml. of saline.

At least one known positive serum and one known negative serum were included with each group of unknown serums. The tubes were shaken and incubated for three hours in a water bath at 37 C. and shaken upon removal. Although the test could be read immediately upon removing the tubes from the water bath, the final reading was made twelve hours later. In reading the tests, each tube was gently shaken to resuspend the cells. The cells in negative tubes were difficult to resuspend but eventually formed a homogeneous suspension after considerable shaking. The agglutinated cells in positive tubes were easily dislodged from the bottom and appeared as clumps in the liquid.

RESULTS

Details of the serological tests are shown in table 1. It is noted that most of the animals artificially sensitized with either *M. paratuberculosis* or *M. tuberculosis* var. *bovis* showed positive titers at 1 : 128, which was the highest titer tested. It was observed that the clumps of erythrocytes were larger in tubes containing serums from Johne's sensitized cattle than they were in the tubes containing serums from tuberculosis sensitized cattle. Without previous knowledge of their identity, an observer was able to correctly differentiate ten out

TABLE 1—Results of Hemagglutination Tests on Serums from Five Groups of Cattle

Source of serums	No. of samples	Titers					
		1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128
Sensitized with *Mycobacterium paratuberculosis*,* positive to intradermal johnin and tuberculin	28	28/28	28/28	27/28	27/28	25/28	24/28
Sensitized with *M. tuberculosis* * *bovis*, positive to intradermal johnin and tuberculin	18	18/18	18/18	17/18	16/18	16 18	16/18
M. paratuberculosis-infected herds	67	60/67	47/67	21/67	10/67	7 67	4/67
M. bovis-infected herd negative to intradermal tuberculin	13	13/13	10/13	6/13	2/13	0 13	0/13
No history of Johne's disease or tuberculosis. All negative to intradermal johnin.	55	5/55	2/55	1/55	0/55	0 55	0/55
Total	181						

* Artificially sensitized by intraperitoneal inoculation of heat killed organisms.

of twelve times between test serums from Johne's and tuberculosis sensitized animals.

The serum titers ranged from two positive at 1:32 to all positive at 1:4 in 13 animals in the herd having a previous history of tuberculosis. In 55 animals in herds with no histories of Johne's disease, it was observed that 5 showed titers of 1:4; 2 of these had titers of 1:8, and 1 had a titer of 1:16 but none had titers of 1:32. It should be noted that the 5 animals showing serum titers were all in the herd which was pastured adjacent to premises having cattle infected with Johne's disease. After studying these results, it was decided that a titer of 1:32 or greater was not likely to be observed in noninfected animals.

The serum titers of the 67 animals in herds known to be infected with Johne's disease ranged from 4 positive at 1:128 to 60 positive at 1:4. These serum titers are correlated with intradermal johnin reactions of the same animals in table 2. Since

TABLE 2—Comparative Results with Serums from Cattle in Herds Known to be Infected with Johne's Disease

Results of tests	No. of Animals
Negative to intradermal johnin and with a hemagglutination titer of 1:16 or less.	28
Positive to intradermal johnin and with a hemagglutination titer of 1:32 or greater.	10
Negative to intradermal johnin and with a hemagglutination titer of 1:32 or greater.	15
Positive to intradermal johnin and with a hemagglutination titer of 1:16 or less.	14
Total	67

a titer of 1:32 was not observed in serums from cattle with no history of Johne's disease, this level was arbitrarily selected as denoting a hemagglutination reactor to Johne's disease, and a 3-mm. increase in skin thickness due to the injection of 0.2 cc. of intradermal johnin was considered a positive reaction. Fifteen of the 67 animals showed a serum titer of 1:32 or greater but were negative to the intradermal johnin test, while 14 showed a titer of 1:16 or less but were positive to the intradermal johnin test. The tests were in agreement on 10 animals which reacted to both, and on 28 which were negative to intradermal johnin and showed a serum titer of 1:16 or less.

DISCUSSION

Since titers were observed in serums of tubercular cattle and also from cattle in a herd from which tuberculin reactors had been removed, the johnin antigen is not species specific; however, it is probably specific for the genus Mycobacterium since positive reactions were not observed in cattle from herds with no history of Johne's disease or tuberculosis. In this experiment, it was difficult to compare the accuracy of the hemagglutination test with the accuracy of the intradermal johnin test since cattle which were positive to one or the other were not available for postmortem diagnoses.

The test could be tried as a supplemental diagnostic procedure in problem Johne's disease herds, or by using the test with a homologous antigen in problem tuberculosis herds.

The test could also be used for making a preliminary survey of herds in which Johne's disease was suspected, since blood samples could be secured by personnel unfamiliar with reading an intradermic johnin test and sent to a laboratory.

The difference observed in the agglutination reactions of serums from animals of two different experimental acid-fast sensitizations suggests the possibility of developing products capable of sensitizing erythrocytes against particular species of Mycobacterium. A study of this phenomenon is in progress.

SUMMARY

Studies made on a hemagglutination test for the diagnosis of Johne's disease in cattle are described with the following observations.

1) A purified protein derivative of johnin was a satisfactory agent for sensitizing sheep erythrocytes for use in the test. However, serums obtained from animals sensitized with *Mycobacterium tuberculosis* var. *bovis* also agglutinated sensitized cells, indicating a cross reaction of species within the genus Mycobacterium.

2) Tests were made on serums from 55 cattle with no history of Johne's disease or tuberculosis; 50 showed no titer, while the titers of 5 ranged from 1:4 to 1:16, with none reaching a titer of 1:32. This range was accepted as indicative of normal animals and a titer of 1:32 or over was arbitrarily selected as denoting a reactor.

3) Serums from 10 intradermal johnin reactors showed titers of 1:32 or over, while the serums of 14 other reactors did not. On the other hand, 15 cattle which showed a titer of 1:32 or more did not react to the intradermal johnin test.

4) Serums from cattle artificially sensitized with *M. paratuberculosis* caused sensitized erythrocytes to form larger clumps than did serums from cattle artificially sensitized with *M. tuberculosis* var. *bovis*.

References

[1] Hagan, W. A., and Zeissig, A.: Studies on the Control of Johne's Disease. J.A.V.M.A., *82*, (1933): 391-407.

[2] Sigurdsson, B., Vigfússon, H., and Theodors, S.: Serological Diagnosis of Johne's Disease of Sheep. J. Comp. Path. Therap., *55*, (1945): 268-276.

[3] Hole, N. H.: Johne's Disease. I. Present-Day Diagnosis and a Preliminary Note on an Investigation into the Value of a Serological Method. Vet. Res., *64*, (1952): 601-603.

[4] Middlebrook, G., and Dubos, R. J.: Specific Serum Agglutination of Erythrocytes Sensitized with Extracts of Tubercle Bacilli. J. Exptl. Med., *88*, (1948): 521-528.

[5] Scott, N. B., and Smith, D. T.: A Simple Modification of the Middlebrook and Dubos Hemagglutination Test for Serum Antibodies to Products of Tubercle Bacilli. J. Lab. and Clin. Med., *35*, (1950): 303-307.

[6] Smith, D. T., and Scott, N. B.: Clinical Interpretation of the Middlebrook-Dubos Hemagglutination Test. Am. Rev. Tuberc., *62*, (1950): 121-127.

[7] Fisher, S., and Gregory, T. S.: Studies on a Serological Test for the Diagnosis of Tuberculosis in Cattle. Austral. Vet. J., *27*, (1951): 25-34.

[8] Fisher, S.: Antigenic Relationships of Erythrocyte Adsorbable Fractions of Some Mycobacteria. Austral. J. Exptl. Biol. and M. Sci., *29*, (1951): 1-8.

[9] Sohier, R., Trimberger, J., and Jullard, J.: Reaction d'hémagglutination type Middlebrook-Dubos réaliseé avec les hématies humaines O et la nouvelle tuberculine I.P. 48. Ann. Inst. Pasteur., *79*, (1950): 347-350.

[10] Sohier, R., Simintzis, G., and Jullard, J.: La réaction d'hémagglutination type Middlebrook-Dubos dans le diagnostic de la tuberculose bovine. Bull. Acad. vét. France., *23*, (1950): 393-397.

[11] Gernez-Rieux, A., Tacquet, A., Gaumont, R., Verge, J., and Couchy, L.: La reaction d'hémagglutination (Réaction de Middlebrook-Dubos) dans la paratuberculose bovine. Bull. Acad. vét. France, *23*, (1950): 465-468.

[12] Green, H. H.: Weybridge P.P.D. Tuberculins. Vet. J., *102*, (1946): 267-278.

A Theoretical Discussion of Ruminant Ketosis

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IN RECENT years we have witnessed significant advances in our understanding of animal physiology. The techniques of enzymology and the use of isotopic tracers have brought us to the point where it is possible, in many instances, to delineate the processes of metabolism as a series of definite chemical reactions. It has become evident that the major metabolites—carbohydrates, fats, and proteins—are degraded to a few simple compounds which enter a common cycle for catabolism and serve as the building blocks for synthesis.

We have also recently arrived at a new understanding of the processes of ruminant digestion. Formerly, it was recognized that cellulose is digested with the aid of bacteria. Aside from the degradation of cellulose to glucose, it was assumed that foodstuffs are digested much as they are in other mammals. It has now been conclusively demonstrated, however, that, unlike other animals, the ruminant is largely dependent upon the products of microbial fermentation and upon the microörganisms themselves for its metabolic needs. Carbohydrates, as such, are not a significant source of energy. Instead, the animal depends largely upon the short-chain organic acids produced from the fermentation of carbohydrates. It has been demonstrated that these acids are absorbed directly through the wall of the rumen. See the reviews by Phillipson [1] and Elsden and Phillipson [2] for a discussion of the development of these concepts.

Several investigators[3-11] have considered the relationships between rumen fermentation and the problem of ketosis. As our background knowledge now appears to be sufficient to predict lines of investigation that should lead to a solution of the problem, the time seems appropriate for a theoretical discussion of ruminant metabolism with a review of recent developments and a consideration of their implications as they apply to ketosis.

THE PRINCIPAL CATABOLIC PATHWAYS

Glucose.—The anaërobic pathway of glucose metabolism has been known for some time. When glucose is first absorbed it may be stored as glycogen by the way of glucose → glucose-6-phosphate → glucose-1,6-diphosphate → glucose-1-phosphate → glycogen. This is broken down as needed to pyruvic acid by the reaction sequence—glycogen → glucose-1-phosphate → glucose-1,6-diphosphate → glucose-6-phosphate → fructose-6-phosphate → fructose-1,6-diphosphate → 3-phosphoglyceraldehyde → 1,3-diphosphoglyceric acid → 3-phosphoglyceric acid → 2-phosphoglyceric acid → phosphoenolpyruvic acid → pyruvic acid. If glucose is needed for storage or synthesis, it may be formed from pyruvic acid by a reversal of these reactions.[12, 13]

Recently, it has been shown that there is also an aërobic pathway for glucose degradation. This leads to the formation of pentose and the further degradation of this pentose to a C_2 fragment, which has not been definitely identified, and triose phosphate, which forms pyruvic acid. The relative importance of the two pathways is not known, although it appears that for energy, rather than synthesis, needs the anaërobic pathway is probably followed.[13-15]

Pyruvic acid may be further oxidatively decarboxylated to acetyl coenzyme A, or it may be carboxylated to malic acid by the reactions shown in figure 1.[15, 16, 17] It will be noted that the formation of malic acid from pyruvic acid is reversible but that the formation of acetyl CoA from pyruvic acid is not.

Fats.— Fats are first hydrolyzed to fatty acids and glycerol. Only recently have we begun to understand the initial "sparking" needed for fatty acid oxidation. In order for the reaction to be initiated it is necessary that oxidation of citric acid cycle intermediates be proceeding so that a continuous supply of adenosine triphosphate may be available as a source of high energy bonds.[18] Green [19] has described the process as following the steps shown in figure 2.

The acyl CoA residue again undergoes successive β oxidation until the acid has been degraded to an acetoacetyl CoA fragment. This last C_4 fragment may be degraded further to acetyl CoA, or it may be hydrolyzed to free acetoacetic acid.[20]

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The following abbreviations are used: CoA for coenzyme A (coenzyme A is known to consist of adenosine-2- or 3-phosphate connected to the thioethanolamide of pantothenic acid by what is probably a pyrophosphate linkage. It apparently activates an acid by the formation of a high energy acyl mercaptide linkage.[18]), dpn and dpn(red) for oxidized and reduced diphosphopyridine nucleotide, tpn and tpn(red) for oxidized and reduced triphosphopyridine nucleotide, atp for adenosine triphosphate, amp for adenosine monophosphate, p-p for inorganic pyrophosphate.

The β oxidation of the activated fatty acid to the β-keto acid is not well understood but is known to be a source of high energy bonds.[21, 28] It will be noted that the initial formation of the acyl CoA is an energy consuming process but that in the ensuing additions of coenzyme A to the activated β-keto acid an activated coenzyme A compound is not required.

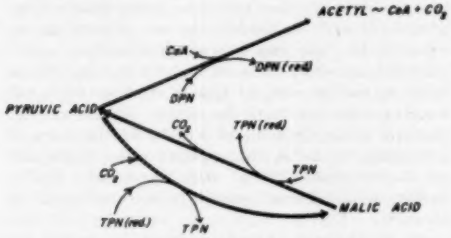

Fig. 1—Pyruvic acid oxidation.

If the acetyl CoA can not be further metabolized at the moment, it can condense with another molecule of acetyl CoA to form acetoacetic acid,[28] which is freely transported to other parts of the body or eliminated in the urine. Acetoacetic acid may be metabolized after being reactivated by pyrophosphoryl CoA and splitting to form acetyl CoA again. It may also be decarboxylated to acetone, although this pathway appears to be followed only to a minor degree.[29] Sakami and Lafaye[24] have reported work which indicates that acetone may be oxidized to pyruvate.

The glycerol portion of fat is oxidized to triose phosphate and may then form pyruvic acid.[25, 26]

Acetic Acid.—Acetic acid is activated to form acetyl CoA by the same mechanism as are the longer fatty acids.[30] It will be noted, however, that this is an energy consuming conversion and that no energy is obtained by a β oxidation as is the case for longer-chain fatty acids.

Propionic Acid.—Propionic acid must likewise be activated before reaction is possible.[27] It is then evidently carboxylated to form succinic acid.[30]

Butyric Acid.—Butyric acid is metabolized in the same way as are the other even-numbered fatty acids. It should be noted, however, that it may be oxidized to acetoacetic acid directly without the formation of acetyl CoA fragments.[30]

Protein.—Protein is hydrolyzed to its constituent amino acids. In many cases these are converted to keto acids by transamination[28] or direct deamination. Citrulline,[29] arginine,[29, 30] ornithine,[30] hydroxyproline,[30] proline,[30] and histidine[31] form glutamic acid, which is converted to α-ketoglutaric acid by transamination.[29] Aspartic acid[29] forms malic acid, and methionine[30] probably forms oxaloacetic acid, although it is not an efficient glycogen former.[30] Alanine, glycine,[32, 34] and serine[36] are converted to pyruvic acid. Valine[36] forms a three-carbon acid, probably propionic acid.[37]

Isoleucine[37, 39] is degraded to propionic acid and acetic acid. Phenylalanine[15] forms tyrosine,[38] which is cleaved to produce malic acid and acetoacetic acid. Tryptophane[15] is degraded to alanine and anthranilic acid. Leucine[39, 40] is degraded to 1 molecule of acetoacetic acid and 1 of acetic acid. It is considered to be one of the most strongly ketogenic amino acids. Lysine[41–43] is apparently degraded to glutaric acid, which is oxidatively decarboxylated to butyric acid. These reactions are summarized in figure 3.

Threonine[44] can form glycogen, as can norleucine,[45] although the pathways followed have not been determined. The cystine and cysteine pathways of catabolism are not known. They appear to form liver glycogen but so inefficiently that the proof is not unequivocal.[32]

The Krebs' Citric Acid Cycle.—At the final stage of oxidation, known as the Krebs' citric acid cycle,[15, 46] diagrammed in figure 4, the products of the degradation of the carbohydrates, fats, and proteins join in the common pathway that is the principal source of energy for the many life functions. Acetyl CoA condenses with oxaloacetic acid to form citric acid, which passes through a series of oxidations which result in the re-formation of oxaloacetic acid. Thus all substances that form acetyl CoA enter the cycle directly; those that form pyruvic acid enter it after degradation to acetyl CoA or are carboxylated to serve as a source of oxaloacetic acid; and those that form dicarboxylic acids may serve as a source of oxaloacetic acid or be oxidized by the pathway malic acid → pyruvic acid → acetyl CoA at which point they may enter the cycle for complete oxidation.

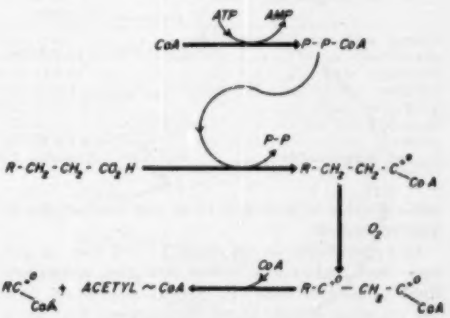

Fig. 2—Fatty acid oxidation.

Oxaloacetic acid is itself subject to oxidation. The reactions that lead to the formation of dicarboxylic acids from amino acids are reversible so that when protein is being synthesized the intermediates of the cycle may be removed for the synthesis of "nonessential" amino acids.[47, 48] A continual replenishment of oxaloacetic acid for the cycle is therefore necessary. Carbohydrates can furnish the necessary intermediates for their own oxidation. Proteins can do likewise. It will be

noted, however, that in the case of fats only the glycerol portion, a small part of the total molecule, is capable of furnishing oxaloacetic acid and that in the case of fatty acids there is no source of oxaloacetic acid.

RUMINANT NUTRITION

Quantity of Glucose Absorbed.—It is not the food that is consumed but that which is actually assimilated that is of importance to the animal. Although it is evident that a large portion of the carbohydrates ingested are converted to volatile fatty acids by the microörganisms of the rumen, a knowledge of the amount that is absorbed as glucose would be useful.

Phillipson and McAnally[49] found only small amounts of glucose in the abomasum of 2 sheep fed 100 Gm. of glucose, while in a third they found none. Heald is reported to have found that the glucose obtained from bacterial polysaccharides passing from the abomasum of sheep fed solely hay does not exceed 6 Gm. in twenty-four hours.[50] McClymont,[51] referring to unpublished work, states: "Data obtained using the Crocker-Markowitz cannula technique indicate that at least on a diet of lucerne hay and green kale the amount of reducing sugar available to a sheep for enzymatic intestinal digestion is equivalent to only 1 to 3 Gm. of glucose per day."

Schambye[50, 52, 53] has concluded, from studies of arterial and portal blood glucose differences, that little glucose is absorbed from any part of the digestive system.

TABLE 1.—Molecular Proportions of Short-Chain Fatty Acids Found in the Rumen

	(%)
Formic acid	0.0 to 5.0
Acetic acid	45.0 to 76.0
Propionic acid	12.0 to 29.0
n-Butyric acid	6.0 to 19.0
iso-Butyric acid	0.3 to 2.8
Branched C_5 acids	0.8 to 5.0
n-Valeric acid	0.6 to 3.3
C_6 and higher acids	Trace to 1.9

In experiments in which 10 to 100 Gm. of glucose were injected directly into the abomasum, Reid[54] observed that the rate of absorption from the intestine is very slow. He suggests that sheep have a low hexokinase activity in the intestinal mucosa.

These varied approaches to the problem all lead to the final conclusion that little glucose is absorbed by the sheep. There is nothing to indicate that such is not also the case for other ruminants.

Quantities of the Short-Chain Acids Absorbed.—Various analyses[55-60] show the range of values for the fatty acids found in the rumen of sheep indicated in table 1. McClymont[51] reports the following ranges in 2 cows: acetic acid 52.3 to 69.0, propionic acid 16.3 to 30.5, butyric acid 10.4 to 18.1, and higher acid 2.6 to 5.1 molecular per cent.

These figures have little significance, however, unless the rates at which the various acids are absorbed through the wall of the rumen are known. One method of studying these rates of absorption is to observe the rates of disappearance of mixtures of the acids from the rumen with time. Using inadequate methods of analysis, Danielli *et al.*[60] calculated that the sodium salts of the acids disappeared at rates—acetate > propionate > butyrate. Gray[61] contended that no absorption occurred when the rumen was alkaline but later[62] modified his views. Masson and Phillipson[63] found that the sodium salts disappear in substantial, but varying, amounts from the rumen. In the case of the free acids, Danielli *et al.*[60] found the rates of absorption to be in the opposite order from that of their salts—butyric acid > propionic acid > acetic acid. These results were confirmed by Gray.[62]

In the normal animal, however, the acids are present, not as the free acid or the sodium salts but as mixtures of intermediate pH. Masson and Phillipson[63] found that the fall in concentration of the rumen contents in an acid rumen (pH 5.5 to 5.9) is more than twice that in an alkaline rumen (pH 8.3 to 8.0).

Using goats, the author,[5] in an attempt to approximate physiological conditions, studied the changes in concentration of the rumen acids that occurred when a mixture of the acids, containing a small quantity of KOH to provide alkali normally furnished in the feed, was dripped into the rumen via a fistula over a period of six hours. In this way, differences in the rates of absorption were easily demonstrated without the necessity of exceeding at any time the normal concentration of volatile acids in the rumen. The conclusion was reached that a very rough estimate of the proportions of the acids produced in the rumen was justified. If the rumen analysis shows about 65 molecular per cent acetic acid, 20 molecular per cent propionic acid, and 15 molecular per cent butyric acid, the amounts actually produced probably are about 60 per cent, 20 per cent, and 20 per cent respectively.

Another approach to the problem is the determination of the differences in concentration between the acids in the blood draining the rumen (adjusted for the concentrations in the arterial blood) and the acids in the rumen itself. Barcroft, McAnally, and Phillipson[64] found that the blood draining the rumen of sheep contains a higher percentage of the lower molecular weight acids than the rumen. Therefore, they concluded that acetic acid is absorbed more rapidly than propionic acid, which is absorbed more rapidly than is butyric acid. From experiments in which the sodium salts of the acids were introduced separately into the washed rumen, they concluded that these are absorbed in the same order. Masson and Phillipson[63] added the sodium salts of the acids individually to the rumen. After making adjustments for the acetic acid in the arterial blood, they

found acetate to pass into the blood more readily than propionate, while only small amounts of butyrate appeared in the blood. At higher concentrations greater variations were observed, but the same order still prevailed.

A comparison of the rates of disappearance of the acids from the rumen with the concentrations found in the blood showed large discrepancies, particularly in the case of butyric acid. It was suggested that the discrepancies were caused by selective absorption in the lymph or by selective metabolism in the wall of the rumen. Kiddle, Marshall, and Phillipson [57] separated the acids of the rumen and the blood draining the rumen of sheep that had been taken directly from pasture. They found that the mixture of acids calculated to enter the blood as it flows through the rumen, after a correction for the acetic acid present in the arterial blood, is similar to that present in the rumen except that there is much less butyric acid. The lymph was found not to be a significant route for absorption of the volatile acids.

Pennington [65] has demonstrated in *in vitro* experiments that butyric acid is selectively metabolized, largely to ketone bodies, by rumen epithelial tissue and that acetic and propionic acids are also metabolized but to a lesser extent. He also demonstrated a marked rise in the ketone bodies in rumen blood during the absorption of butyrate. The apparent discrepancies between the two methods are thereby reconciled, and the conclusions reached from rate of disappearance studies appear valid.

Yet another method of determining the relative amounts of acids actually produced in the rumen is by *in vitro* fermentation. The percentage values obtained by Gray, Pilgrim, and Weller [56] from the rumen contents of sheep fed wheaten hay were acetic acid 46.0, propionic acid 41.0, and butyric acid 13.0; while from the rumen contents of sheep fed lucerne hay, acetic acid 59.0, propionic acid 27.0, and butyric acid 14.0 were the proportions obtained. It is doubtful, however, if a duplication of rumen fermentation was obtained as the substrates available to the different organisms were different from those found in the rumen due to the absence of absorption. Gray *et al.*[58] have, in fact, themselves presented evidence which leads one to question their conclusions. Using labelled acetic and propionic acids, they have shown that carbon atoms are exchanged between the various acids.

In an *in vitro* system, where the products of fermentation accumulate, these interconversions would be expected to differ in degree from those occurring in the rumen itself. Recognizing that evidence obtained from the animal in the normal state would be more conclusive, Gray and Pilgrim [60] made a study of the changes in fatty acid concentration in the rumen during the course of digestion. Assuming that the rate of production is constant, they interpreted the variations in proportions of the acids occurring as resulting from differences in the rates of absorption. From their observations, they concluded that propionic acid is absorbed more rapidly than either acetic or butyric acid. The assumption that the rate of production is constant hardly seems warranted. It is known that the more soluble carbohydrates are attacked more rapidly than are the less soluble ones. Thus the substrate obviously changes with the progress of fermentation. It would seem reasonable that variations would occur due to the increase in activity of microörganisms that use as a substrate the products of other microörganisms. For example, the production of propionic acid might be delayed until lactic acid was being produced by other organisms.

The data obtained by rate of disappearance of the acids from the rumen thus appears to be the most reliable now available.

It should be noted that the proportions of the volatile acids found in the rumen are presented as molecular percentages. Kiddle, Marshall, and Phillipson [57] have pointed out that the caloric values have been reported to be 3.49, 4.96, and 5.95 kg. cal./Gm. for acetic, propionic, and butyric acids respectively. If the molecular percentages of the acids produced in the rumen are 60.0 acetic, 20.0 propionic, and 20.0 butyric, the relative contributions to energy production are 41 per cent, 24 per cent, and 35 per cent.

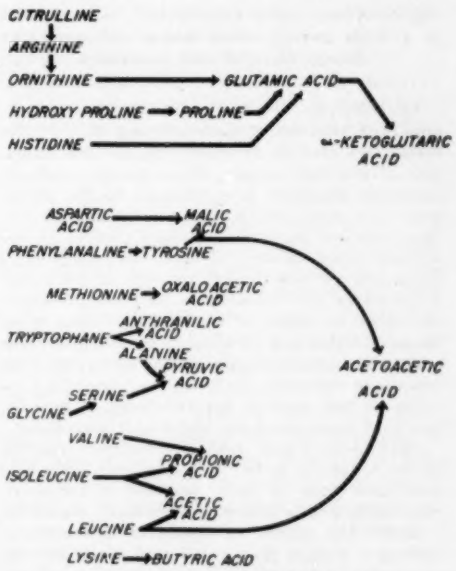

Fig. 3—Amino acid oxidation.

Digestion of Fats and Proteins.—The fats and proteins of the diet are also used by the rumen microörganisms for the satisfaction of their metabolic needs. The demonstration that when suffi-

cient carbohydrate is available to supply the carbon and energy needed for synthesis urea is readily converted to protein clearly shows the versatility of rumen digestion. Shazly [66] has shown that when proteins are present in excess of the structural needs of the microörganisms they are readily deaminated and used for energy purposes with the production of fatty acids.

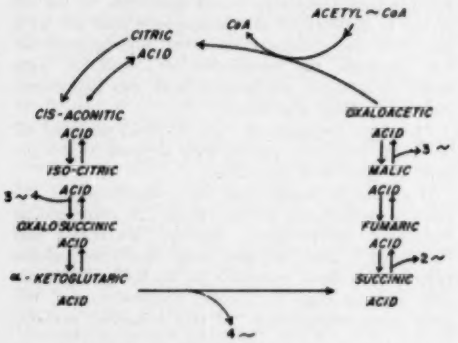

Fig. 4—Krebs' citric acid cycle. ~ — formation of a high energy bond which furnishes the energy for synthetic processes.

The difference between the use of fats and proteins and the use of carbohydrates is that the former are used by microbial life for the formation of cell tissue, while the latter are used, by means of anaërobic fermentation, as the energy source for synthesis and the maintenance of life. The inefficient anaërobic processes produce as end products large amounts of short-chain fatty acids which are removed through the wall of the rumen and used by the ruminant as its principal source of energy by means of its efficient aërobic processes. The fats and proteins of the cell structures are made available to the animal after they have undergone enzymatic digestion.

Let us now examine our knowledge of how the ruminant metabolizes the material it assimilates.

Short-Chain Fatty Acid Metabolism.—Analyses of the peripheral blood [61, 67, 68] show that acetic acid constitutes from 87 to 97 per cent of the short-chain acids and is present in significant quantities.

Reid [67] has shown, by means of arteriovenous difference studies, that acetic acid is metabolized in significant quantities by the tissues of the sheep's head. McClymont,[68] also using arteriovenous difference studies, has shown that acetate is metabolized in the mammary gland. Popják [70] has discussed the importance of acetate in mammary gland metabolism in a recent review. Schultz, Smith, and Lardy [10, 71] have reported that acetic acid causes a rise in neither the blood sugar nor blood ketone bodies in goats. Jarrett and Potter [7] reported experiments which showed acetate to be somewhat ketogenic. No rise in blood pyruvic or lactic acid was found.

Acetate is thus used readily for the synthesis of fats and as a source of energy by oxidation via the citric acid cycle.

A comparison of the proportion of propionic acid in the blood draining the rumen reported by Kiddle *et al.*[57] and that found in the arterial circulation indicates that most of the propionic acid is metabolized in the liver. The arteriovenous difference studies reported by Reid [67] indicate that little propionic acid is removed during peripheral circulation. Propionic acid is undoubtedly glucogenic in the ruminant.[10, 67, 72] Jarrett and Potter [7] have shown that propionic acid causes a rise in blood pyruvic and lactic acids and a depression in the level of total ketone bodies. This body of evidence indicates that propionic acid is converted to glucose in the liver and then is directed into the peripheral circulation as needed.

The previous discussion has shown that butyric acid is produced in significant amounts in the rumen and oxidized to ketone bodies in its wall before entry to the blood stream. Pennington [66] has also shown that these ketone bodies appear in the arterial blood. Shaw and Peterson [73] have reported that β-hydroxybutyric acid is readily utilized by the mammary tissue. Schultz and his coworkers [10, 71] have shown that butyric acid is ketogenic in goats. In unpublished work, the author has noted that this ketogenic effect occurs even when glucose is given concurrently.

Our understanding of the effect of butyric acid upon the blood glucose is indeed in a state of confusion. Schultz and Smith [10] have quoted cases in which goats given butyric acid demonstrated an abrupt drop in the blood sugar level, giving a blood picture typical of a clinical case of ketosis. They found that acetoacetic acid given orally as the sodium salt caused a rise in blood sugar, probably because of a toxic effect, while β-hydroxybutyric acid and acetone caused no significant changes. The author, having observed this effect in goats and cows stated: "When equal amounts of propionic acid and butyric acid are received by the animal the rise in blood glucose is far less than if the same quantity of propionic acid alone is received."[74] It has since been observed that this is not invariably the case. In some goats, butyric acid seems to show no glucose-depressing action when given alone or in conjunction with propionic acid. Recently, Potter [74] reported experimental work on young lambs rendered hypoglycemic with insulin. Intravenous injections of sodium butyrate or sodium propionate relieved the convulsions but equimolar amounts of acetate did not. The increase in total reducing substances in the blood was greater and more prolonged in the case of butyrate than of propionate. Acetate caused no increase. The author has given butyrate repeatedly to starved goats. High glucose levels were noted when a high blood carbon dioxide capacity indicated a state of acute alkalosis. This was interpreted as being a result of anoxia. The fact that acetate did not cause a similar result indicates that in Potter's work alkalosis was not a factor.

Further investigation of this problem is obviously desirable.

The work on the effects of the individual volatile acids upon ruminants is enlightening. Under normal conditions, however, the animal is metabolizing a combination of these acids. Jarrett and Potter[7] have reported that an equimolar mixture of acetate and propionate produces no rise in ketone bodies in the blood while acetate alone causes a marked rise and propionate alone causes a marked depression. It was also shown that acetate plus propionate causes much less increase in blood pyruvic acid than does propionate alone. The author, in as yet unpublished work, has observed that propionic acid has a similar depressing action on the ketogenic effect of butyric acid. Quastel and Wheatley[75] have observed a similar antiketogenic action of propionic acid on butyric acid in *in vitro* studies with liver slices. This effect might be interpreted as being due to the prevention of oxidation of butyric acid by the competition of propionic acid for the same enzymes. If large quantities of the two acids are given, however, a rise in blood sugar and ketone bodies above normal levels will be noted. Thus, both substances are metabolized. A possible explanation is that propionic acid, as noted in the section on catabolic mechanisms, must form intermediates of the citric acid cycle. This would facilitate the complete oxidation of butyric acid and, until very large amounts are administered, thus prevent a rise in blood ketone bodies. The author has also observed that, although acetic acid seems to have little ketogenic action when administered alone, when given in equimolar quantities with butyric acid it markedly accentuates the ketogenic effect of the butyric acid. This effect also seems reasonable, in the light of the discussion on mechanisms, as the acetic acid furnishes enough acetyl CoA fragments for the citric acid cycle so that there should be no tendency for butyric acid to be oxidized beyond acetoacetic acid, in which form it may be eliminated.

This summary of the studies of the metabolism of the short-chain fatty acids in ruminants furnishes ample evidence that these animals use the same mechanisms for the life processes as do the animals used for the elucidation of the pathways of metabolism discussed in the first section of this paper. The differences found in the ruminant are differences in degree but not in fundamental metabolic reactions.

The ruminant, unlike many other animals, assimilates little glucose. The volatile fatty acids are the principal source of energy. The low carbohydrate tolerance[67, 76–78] and the facility with which acetic acid is metabolized confirm this. Particularly convincing is the demonstration that the ruminant mammary gland uses acetate much more readily than do the mammary glands of other species.[70]

There is no evidence that the ruminant uses fats or amino acids in ways differing from other animals.

In the light of the mechanisms of metabolism and the special aspects of rumen digestion outlined above, let us examine the problem of ruminant ketosis.

KETOSIS

The dependence of the ruminant on the short-chain fatty acids for most of its energy places it in a peculiar position in respect to its susceptibility to ketosis. The largest portion of carbohydrate in its diet is converted to acetic and butyric acids, which can not form glucose but can form ketone bodies. Butyric acid forms ketone bodies readily even in the presence of sufficient glucose. Approximately one fifth of the acids produced, however, is propionic acid. This acid can not only form glucose in the body but has greater antiketogenic activity than glucose itself, being able to counteract even the ketogenic activity of butyric acid. It is evident that the balance of such a system must be precarious—slight changes in the proportions of these acids can cause large shifts in the ketogenic-antiketogenic balance.

We have long known that the ketotic ruminant has a low blood sugar level. We have also known that intravenous glucose will relieve the condition. Now that it is understood that oral carbohydrate is a poor source for glucose, it seems fairly evident that it is a lack of sufficient glucose for synthetic needs that brings about the disturbance.

One effect of a glucose deficiency is the use of oxaloacetic acid for the synthesis of the needed glucose. As oxaloacetic acid acts in a catalytic capacity in the operations of the citric acid cycle, a shortage has a severe effect on the energy economy of the animal.

If oxaloacetic acid is absent acetic acid, normally one of the principal sources of energy to the ruminant, becomes of no value. Nothing has been published on the excretion of acetic acid by the ketotic ruminant. It would be interesting to know whether it can be eliminated as such or whether it must first be condensed to acetoacetic acid. If the latter is the case, acetic

acid is not only valueless but is a liability, as the formation of acetoacetic acid from acetic acid is an energy-consuming process.

The oxidation of butyric acid to acetoacetic acid will furnish a small amount of energy, although a portion of this energy must be used for activation of the acid. The blood sugar-depressing action is not easily evaluated as its theoretical explanation is not apparent.

Propionic acid becomes important to the animal as it is a potential source of the catalytic amounts of oxaloacetic acid needed. If the supply is insufficient, however, the oxaloacetate formed is quickly drained away for the synthesis of glucose.

Fats, derived from the diet and from the body reserves, can be used for energy. As was pointed out previously, the glycerol portion is available for the formation of glucose or pyruvate. The fatty acid portion will furnish a substantial amount of energy through oxidation to acetyl CoA. The final pathway for the oxidation of the acetyl CoA fragments is inadequate, however, so a certain portion of them, depending on the degree of oxaloacetic acid deficiency, form acetoacetate. The ability to degrade fats without a concurrent oxidation of the 2-carbon residue appears to reside primarily in the liver.[79] The acetoacetate formed can be used to a certain extent by the peripheral tissues if they have oxaloacetic acid available, while that which can not be utilized is excreted by the kidneys.

In most animals, when exogenous carbohydrate is lacking, proteins are degraded to supply the demand. The discussion on catabolic mechanisms indicated that many amino acids follow pathways that can lead to the synthesis of glucose. This process, however, involves first a deamination and the formation of urea. McCandless and Dye,[76, 77, 80, 81] with their co-workers, have shown that under conditions of glucose shortage the ruminant exhibits little increase in the excretion of nitrogen. They suggest that this pathway of gluconeogenesis is of subordinate value to the ruminant.

In ketosis, we thus have a situation in which fat is desperately and wastefully spent for the small supply of glucose and energy which it can furnish without citric acid cycle oxidation. The residue, acetoacetic acid, is excreted in the urine as an alkaline salt thus causing acidosis.

Although it seems reasonably certain that the ketotic ruminant is not receiving sufficient glucose, we shall not know the etiology of the disease until the reason for this inadequacy is explained. It is obvious that the supply is adequate for the many unaffected animals.

There is no doubt that the lactating ruminant has a greater basic need for glucose than it does normally. The lactose and glycerol portion of the fats in milk must be supplied by synthesis from glucose. There may be an active synthesis of "non-essential" amino acids, for the milk proteins, which will constitute a further drain on glucose.

In addition to these legitimate needs, it may be that the ketotic animal is wasteful of glucose. The pathway for the oxidation of glucose, when there is a sufficient supply for use as a source of energy as well as for fulfilling the needs for synthesis, is by way of the oxidative decarboxylation of pyruvic acid to acetyl CoA. If the use of this pathway persists, even in the presence of a shortage of glucose and a surplus of acetyl CoA fragments derived from fat, it will be wasteful. The fact that the glucose tolerance[82, 83] of ketotic cows does not vary greatly from that of normal cows would indicate that the mechanisms for glucose conservation are operating.

It is possible that in the ketotic animal the quantity of propionic acid produced in the rumen is less than is the case in the normal one.

It is also possible that the ability to deaminate amino acids exhibits considerable individual variation. The author, in unpublished work, has observed that a protein hydrolysate given intravenously to normal cows will cause a rise of blood sugar to levels that are above normal, while in the case of 2 ketotic cows no significant rise occurred. Cortisone has been shown to be effective in the treatment of ketosis.[84, 85] Welt *et al.*[86] have shown that cortisone causes a very large increase in the rate of formation of glucose from non-carbohydrate sources. If a deficiency in gluconeogenesis from protein does exist, the reason why some animals succumb to the disorder and others do not becomes apparent.

The normal ruminant may have the ability to overcome the stress caused by a lack of sufficient glucose while the ketotic ani-

mal does not. The ideal treatment for ketosis is not one which enables the animal to meet the stress but one which eliminates that stress. The use of protein to form glucose is fundamentally an uneconomic and wasteful process used by the animal to overcome a metabolic crisis. A more desirable solution would be to furnish the animal with a greater quantity of propionic acid, its natural source of glucose, or with other substances which are efficiently glucogenic.

Progress is being made toward this goal. The author [9] has used tripropionin and glycerol for this purpose. Seekles [87] has reported that ammonium lactate is effective. Schultz [11] has reported success with sodium propionate. None of these solutions is ideal. For an animal already deaminating protein to its maximum ability, ammonium salts would appear to be contraindicated. Alkali salts are probably beneficial in small quantities when treatment is initiated, but the use of large quantities or prolonged use of lesser amounts will probably lead to an undesirable alkalosis. Tripropionin is not water soluble and not palatable. Glycerol is effective but currently expensive.

There are a number of other compounds that will warrant screening. The author is currently testing propylene glycol with results that are encouraging. This material is glucogenic itself but may very possibly be fermented to propionic acid before it is assimilated. It would be of interest to observe the effect of various pentose carbohydrates as these are probably degraded to 2- and 3-carbon fragments by the rumen bacteria.[15]

Any pure chemical compound, while it may be economically feasible for curing or preventing disease, is very likely to be an expensive feed. The spread shown in the reported percentages of volatile fatty acids found in the rumen indicate, however, that variations in the proportions of the acids produced occur naturally. At present, we do not understand what causes these variations. Phillipson [88] has recently described a diet that causes sheep to show a very unusual rumen acid composition. The ultimate solution to the problem of ketosis seems to lie in learning enough about rumen fermentation to control the proportions of the volatile fatty acids produced.

References

[1] Phillipson, A. T.: Fermentation in the Alimentary Tract and the Metabolism of the Derived Fatty Acids. Nutr. Abstr. Rev., *17*, (1947-48): 12-18.

[2] Elsden, S. R., and Phillipson, A. T.: Ruminant Digestion. Ann. Rev. Biochem., *17*, (1948): 705-726.

[3] Duncan, C. W., Huffman, C. F., and Tobin, H. A.: A Chemical Study of Ketosis in a Dairy Herd. J.A.V.M.A., *95*, (1939): 690-700.

[4] Dye, J. A., and McCandless, E. L.: A Physiological Approach to the Problem of Ketosis. I. Basic Features of the Metabolic Processes Concerned in Ketosis. Cornell Vet., *38*, (1948): 331-340.

[5] Hoflund, S., and Hedstrom, H.: Disturbances in Rumen Digestion as a Predisposing Factor in the Appearance of Acetonemia. Cornell Vet., *38*, (1948): 405-417.

[6] Holmes, J. R.: Discussion. Vet. Rec., *62*, (1950): 724-725.

[7] Jarrett, I. G., and Potter, B. J.: Metabolism of Acetate and Propionate in the Ruminant. Nature, *166*, (1950): 515-517.

[8] Johnson, R. B.: The Relative Rates of Absorption of the Volatile Acids from the Rumen and Their Relationship to Ketosis. Cornell Vet., *41*, (1951): 115-121.

[9] Johnson, R. B.: New Methods of Treating Ketosis. A Preliminary Report. North Am. Vet., *32*, (1951): 327-332.

[10] Schultz, L. H., and Smith, V. R.: Experimental Alteration of the Sugar and Ketone Levels of the Blood of Ruminants in Relation to Ketosis. J. Dai. Sci., *34*, (1951): 1191-1199.

[11] Schultz, L. H.: Treatment of Ketosis in Dairy Cattle with Sodium Propionate. Cornell Vet., *42*, (1952): 148-155.

[12] Leloir, L. F.: The Metabolism of Hexose-phosphates. Phosphorus Metabolism, I. Johns Hopkins Press, Baltimore, Md., (1951): 67-93.

[13] Glass, B.: A Summary of the Symposium. Phosphorus Metabolism, I. Johns Hopkins Press, Baltimore, Md., (1951): 658-741.

[14] Horecker, B. L.: The Metabolism of Pentose and Triose Phosphates. Phosphorus Metabolism, I. Johns Hopkins Press, Baltimore, Md., (1951): 117-144.

[15] Ochoa, S., and Stern, J. R.: Carbohydrate Metabolism. Ann. Rev. Biochem., *21*, (1952): 547-602.

[16] Ochoa, S.: Biological Mechanisms of Carboxylation and Decarboxylation. Physiol. Rev., *31*, (1951): 56-106.

[17] Littlefield, J. W., and Sanadi, D. R.: Role of Coenzyme A and Diphosphopyridine Nucleotide in the Oxidation of Pyruvate. J. Biol. Chem., *199*, (1952): 65-70.

[18] Cross, R. J., Taggart, J. V., Covo, G. A., and Green, D. E.: Studies on the Cyclophorase System. VI. The Coupling of Oxidation and Phosphorylation. J. Biol. Chem., *177*, (1949): 655-678.

[19] Green, D. E.: Integrated Enzyme Activity in Soluble Extracts of Heart Muscle. Science, *115*, (1952): 661-665.

[20] Bloch, K.: Interrelationships of Lipid and Carbohydrate Metabolism. Ann. Rev. Biochem., *21*, (1952): 273-300.

[21] Lardy, H.: Discussion. Phosphorus Metabolism, I. Johns Hopkins Press, Baltimore, Md., (1951): 387-388.

[22] Copenhaver, J. H., and Lardy, H. A.: Oxidative Phosphorylations: Pathways and Yield in Mitochondrial Preparations. J. Biol. Chem., *195*, (1952): 225-238.

[23] Stadtman, E. R., Doudoroff, M., and Lipmann, F.: The Mechanism of Acetoacetate Synthesis. J. Biol. Chem., *191*, (1951): 377-382.

[24] Sakami, W., and Lafaye, J. M.: The Metabolism of Acetone in the Intact Rat. J. Biol. Chem., *193*, (1951): 199-203.

[25] Doerschuk, A. P.: Some Studies on the Metabolism of Glycerol-1-C^{14}. J. Biol. Chem., *193*, (1951): 39-44.

[26] Doerschuk, A. P.: Mechanism Studies of Glycogen and Glyceride Glycerol Biosynthesis. J. Biol. Chem., *196*, (1952): 423-426.

[27] Barker, H. A.: Recent Investigations on the Formation and Utilization of Active Acetate. Phosphorus Me-

tabolism, I. Johns Hopkins Press, Baltimore, Md., (1951): 204-245.

[28] Tarver, H.: Metabolism of Amino Acids and Proteins. Ann. Rev. Biochem., *21*, (1952): 301-332.

[29] Ratner, S.: Urea Formation. Phosphorus Metabolism, I. Johns Hopkins Press, Baltimore, Md., (1951): 601-619.

[30] Stetten, M. R.: Mechanism of the Conversion of Ornithine into Proline and Glutamic Acid *in vivo*. J. Biol. Chem., *189*, (1951): 499-507.

[31] Abrams, A., and Borsook, H.: The Conversion of L-Histidine to Glutamic Acid by Liver Enzymes. J. Biol. Chem., *198*, (1952): 205-214.

[32] Hess, W. C.: The Rates of Absorption and the Formation of Liver Glycogen by Methionine, Cystine, and Cysteine. J. Biol. Chem., *181*, (1949): 23-30.

[33] Sakami, W.: The Conversion of Glycine into Serine in the Intact Rat. J. Biol. Chem., *178*, (1949): 519-520.

[34] Barnet, H. N., and Wick, A. W.: The Formation of Glycogen from C^{14}-Labeled Glycine. J. Biol. Chem., *185*, (1950): 657-661.

[35] Chargaff, E., and Sprinson, D. B.: Studies on the Mechanism of Deamination of Serine and Threonine in Biological Systems. J. Biol. Chem., *151*, (1943): 273-280.

[36] Fones, W. S., Waalkes, T. P., and White, J.: The Conversion of L-Valine to Glucose and Glycogen in the Rat. Arch. Biochem. Biophys., *32*, (1951): 89-95.

[37] Coon, M. J., Abrahamsen, N. S. B., and Greene, G. S.: The Relation of α-Methylbutyrate to Isoleucine Metabolism. II. Propionate Formation. J. Biol. Chem., *199*, (1952): 75-84.

[38] Coon, M. J., and Abrahamsen, N. S. B.: The Relation of α-Methylbutyrate to Isoleucine Metabolism. I. Acetoacetate Formation. J. Biol. Chem., *195*, (1952): 805-812.

[39] Coon, M. J.: The Metabolic Fate of the Isopropyl Group of Leucine. J. Biol. Chem., *187*, (1950): 71-82.

[40] Zabin, I., and Bloch, K.: Studies on the Utilization of Isovaleric Acid in Cholesterol Synthesis. J. Biol. Chem., *192*, (1951): 267-273.

[41] Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H.: The Degradation of L-Lysine in Guinea Pig Liver Homogenate: Formation of α-Aminoadipic Acid. J. Biol. Chem., *176*, (1948): 1383-1393.

[42] Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H.: The Degradation of α-Aminoadipic Acid in Guinea Pig Liver Homogenate. J. Biol. Chem., *176*, (1948): 1395-1400.

[43] Rothstein, M., and Miller, L. L.: The Metabolism of Glutaric Acid-1,5-C^{14}. I. In Normal and Phlorhizinized Rats. J. Biol. Chem., *199*, (1952): 199-205.

[44] Hess, W. C.: The Rates of Absorption of and the Formation of Liver Glycogen by DL-Serine and DL-Threonine. J. Am. Chem. Soc., *72*, (1950): 1407.

[45] Butts, J. S., Blunden, H., and Dunn, M.: Studies in Amino Acid Metabolism. III. The Fate of *dl*-Leucine, *dl*-Norleucine, and *dl*-Isoleucine in the Normal Animal. J. Biol. Chem., *120*, (1937): 289-295.

[46] Krebs, H. A.: The Tricarboxylic Acid Cycle. Harvey Lectures Ser., *44*, (1948-49): 165-199.

[47] Recknagel, R. O., and Potter, V. R.: Mechanism of the Ketogenic Effect of Ammonium Chloride. J. Biol. Chem., *191*, (1951): 263-275.

[48] Black, A. L., Kleiber, M., and Smith, A. H.: Carbonate and Fatty Acids as Precursors of Amino Acids in Casein. J. Biol. Chem., *197*, (1952): 365-370.

[49] Phillipson, A. T., and McAnally, R. A.: Studies on the Fate of Carbohydrates in the Rumen of the Sheep. J. Exptl. Biol., *19*, (1942): 199-214.

[50] Schambye, P.: Volatile Acids and Glucose in Portal Blood of Sheep. I. Nord. Vet.-med., *3*, (1951): 555-574.

[51] McClymont, G. L.: Identification of the Volatile Fatty Acid in the Peripheral Blood and Rumen of Cattle and the Blood of Other Species. Austral. J. Agric. Res., *2*, (1951): 92-103.

[52] Schambye, P.: Volatile Acids and Glucose in Portal Blood of Sheep. II. Sheep Fed Hay and Hay Plus Crushed Oats. Nord. Vet.-med., *3*, (1951): 748-762.

[53] Schambye, P.: Volatile Acids and Glucose in Portal Blood of Sheep. III. The Influence of Orally Administered Glucose. Nord. Vet.-med., *3*, (1951): 1003-1014.

[54] Reid, R. L.: Studies on the Carbohydrate Metabolism of Sheep. V. The Effect of Hyperglycemia and of Insulin on the Rate of Extrahepatic Glucose Assimilation. Austral. J. Agric. Res., *3*, (1952): 160-167.

[55] Elsden, S. R.: The Fermentation of Carbohydrates in the Rumen of the Sheep. J. Exptl. Biol., *22*, (1945-46): 51-62.

[56] Gray, F. V., Pilgrim, A. F., and Weller, R. A.: Fermentation in the Rumen of the Sheep. I. The Production of Volatile Fatty Acids and Methane During the Fermentation of Wheaten Hay and Lucerne Hay *in vitro* by Microörganisms from the Rumen. J. Exptl. Biol., *28*, (1951): 74-82.

[57] Kiddle, P., Marshall, R. A., and Phillipson, A. T.: A Comparison of the Mixtures of Acetic, Propionic and Butyric Acids in the Rumen and in the Blood Leaving the Rumen. J. Physiol., *113*, (1951): 207-217.

[58] Gray, F. V., Pilgrim, A. F., Rodda, H. J., and Weller, R. A.: Fermentation in the Rumen of the Sheep. IV. The Nature and Origin of the Volatile Fatty Acids in the Rumen of the Sheep. J. Exptl. Biol., *29*, (1952): 57-65.

[59] Shazly, K. el-: Degradation of Protein in the Rumen of the Sheep. I. Some Volatile Fatty Acids, Including Branched-Chain Isomers, Found *in vitro*. Biochem. J., *51*, (1952): 640-647.

[60] Danielli, J. F., Hitchcock, M. W. S., Marshall, R. A., and Phillipson, A. T.: The Mechanism of Absorption from the Rumen as Exemplified by the Behavior of Acetic, Propionic and Butyric Acids. J. Exptl. Biol., *22*, (1945): 75-84.

[61] Gray, F. V.: The Absorption of Volatile Fatty Acids from the Rumen. II. The Influence of pH on Absorption. J. Exptl. Biol., *25*, (1948): 135-144.

[62] Gray, F. V., and Pilgrim, A. F.: Fermentation in the Rumen of the Sheep. II. The Production and Absorption of Volatile Fatty Acids During the Fermentation of Wheaten Hay and Lucerne Hay in the Rumen. J. Exptl. Biol., *28*, (1951): 83-90.

[63] Masson, M. J., and Phillipson, A. T.: The Absorption of Acetate, Propionate and Butyrate from the Rumen of Sheep. J. Physiol., *113*, (1951): 189-206.

[64] Barcroft, J., McAnally, R. A., and Phillipson, A. T.: Absorption of Volatile Acids from the Alimentary Tract of the Sheep and Other Animals. J. Exptl. Biol., *20*, (1944): 120-129.

[65] Pennington, R. J.: The Metabolism of Short-Chain Fatty Acids in the Sheep. I. Fatty Acid Utilization and Ketone Body Production by Rumen Epithelium and Other Tissues. Biochem. J., *51*, (1952): 251-258.

[66] Shazly, K. el-: Degradation of Protein in the Rumen of the Sheep. II. The Action of Rumen Microörganisms on Amino Acids. Biochem. J., *51*, (1952): 647-653.

[67] Reid, R. L.: Studies on the Carbohydrate Metabolism of Sheep. II. The Uptake by the Tissues of Glucose and Acetic Acid from the Peripheral Circulation. Austral. J. Agric. Res., *1*, (1950): 338-354.

[68] Craine, E. M., and Hansen, R. G.: The Short-Chain Fatty Acids of the Peripheral Blood of Goats. J. Dai. Sci., *35*, (1952): 631-636.

[69] McClymont, G. L.: Volatile Fatty Acid Metabolism of Ruminants, with Particular Reference to the Lactating Bovine Mammary Gland and the Composition of Milk Fat. Austral. J. Agric. Res., *2*, (1951): 158-180.

[70] Popják, G.: The Metabolism of Fat in the Mammary Gland and Fetal Tissues, with Reference to the Application of Isotopic Tracers. Nutr. Abstr. Rev., *21*, (1952): 535-553.

[71] Schultz, L. H., Smith, V. R., and Lardy, H. A.: The Effect of the Administration of Various Fatty Acids on the Blood Ketone Levels of Ruminants. J. Dai. Sci., *32*, (1949): 817-822.

[72] Reid, R. L.: Studies on the Carbohydrate Metabolism of Sheep. IV. Hypoglycaemic Signs and Their Relationship to Blood Glucose. Austral. J. Agric. Res., *2*, (1951): 146-157.

[72] Shaw, J. C., and Petersen, W. E.: The Utilization of β-Hydroxybutyric Acid by the Perfused Lactating Mammary Gland. J. Biol. Chem., *147*, (1943): 639-643.

[74] Potter, B. J.: Relief of Hypoglycaemic Convulsions with Butyric Acid. Nature, *170*, (1952): 541.

[73] Quastel, J. H., and Wheatley, A. H. M.: Oxidation of Fatty Acids in the Liver. Biochem. J., *27*, (1933): 1753-1762.

[70] McCandless, E. L., Woodward, B. A., and Dye, J. A.: Alloxan Diabetes in Sheep under Fasting and Non-fasting Conditions. Am. J. Physiol., *154*, (1948): 94-106.

[77] McCandless, E. L., and Dye, J. A.: Physiological Changes in Intermediary Metabolism of Various Species of Ruminants Incident to Functional Development of Rumen. Am. J. Physiol., *162*, (1950): 434-446.

[79] Holmes, J. R.: Carbohydrate Metabolism in the Bovine. I. Intravenous Glucose Tolerance in the Healthy Cow. J. Comp. Path. Therap., *61*, (1951): 1-14.

[75] Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry. Interpretations. I. Williams and Wilkins, Baltimore, Md., (1946): 440.

[80] Cook, E. T., Dye, J. A., and McCandless, E. L.: Pancreatic Diabetes in the Calf. Am. J. Physiol., *156*, (1949): 349-354.

[81] McCandless, E. L., and Dye, J. A.: Effects of Alloxan Administration in the Calf. Am. J. Physiol., *156*, (1949): 355-360.

[82] Shaw, J. C.: Studies on Ketosis in Dairy Cattle. V. The Development of Ketosis. J. Dai. Sci., *26*, (1943): 1079-1090.

[85] Holmes, J. R.: Carbohydrate Metabolism in the Bovine. II. Intravenous Glucose Tolerance in Various Diseases, Including Clinical Ketosis. J. Comp. Path. Therap., *61*, (1951): 15-25.

[84] Shaw, J. C.: Studies on Ketosis in Dairy Cattle. IX. Therapeutic Effect of Adrenal Cortical Extracts. J. Dai. Sci., *30*, (1947): 307-311.

[83] Shaw, J. C., Hatziolos, B. C., and Chung, A. C.: Studies on Ketosis in Dairy Cattle. XV. Response to Treatment with Cortisone and ACTH. Science, *114*, (1951): 574-576.

[86] Welt, I. D., Sretten, D., Jr., Ingle, D. J., and Morley, E. H.: Effect of Cortisone upon Rates of Glucose Production and Oxidation in the Rat. J. Biol. Chem., *197*, (1952): 57-66.

[87] Seekles, L.: A Note on the Treatment of Ketosis in Milk Cows with Ammonium Lactate. Vet. Rec., *63*, (1951): 494.

[88] Phillipson, A. T.: The Fatty Acids Present in the Rumen of Lambs Fed on a Flaked Maize Ration. Brit. J. Nutr., *6*, (1952): 190-198.

The Nerve Control of Rumination and Reticulo-Ruminal Motility

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THIS STUDY has been undertaken in order to elucidate some of the more obscure facts concerning the nervous control of rumination and reticulo-ruminal motility. It is quite probable that a greater knowledge of the nervous system will help us to better understand the actions of ruminatorics and the complex problems of bloat.

In the course of this problem, we used 63 animals. This includes 7 goats, 2 cows, and 54 sheep. All of these animals were thoroughly checked for diseases and internal parasites before being used for any experiments.

A thorough study of reticulo-ruminal motility and the amount of time spent eating, resting, and ruminating per day was made. Later these same sheep were subjected to various experiments and the deviations from normal were noted.

Motility recordings were made with balloons placed in various parts of the stomach similar to the techniques used by Phillipson,[1] Shalk and Amadon,[2] and others. From these recordings, the normal sequence of events in the reticulum and rumen could be checked. We also made measurements of the time it took for the various parts of the reticulum and rumen to contract. These measurements were made directly from the graphs recorded by the balloon technique. By visual examination and the use of a stop watch, we came to the conclusion that the actual movements of the stomach varied less than ± 0.1 second from those recorded on the graphs. At any rate, the error seemed to be constant, and the graphs made on one day would be very similar to those made on subsequent days.

Table 1 gives the data collected on 6 sheep that were randomly selected from a population of normal sheep. It is assumed that each group of observations represents a random sampling from each sheep. The data (table 1) indicate that the standard error of the sample mean of any given sheep is relatively small (the largest one being about 1.2 in the case of sheep 4); for the purposes of analysis, the given sample means may be regarded as the true means of the sheep. A 95 per cent confidence interval for the mean, m, of the sheep means is given in table 1. Also given is a tolerance interval, which may be interpreted as follows: We are 95 per cent sure that at least 95 per cent of the means of sheep in our original population lie in the given interval.

The Motor and Sensory Nerves to the Reticulum and Rumen

The extensive researches of Mangold and Klein[4] and later Hoflund[5,6] leave little doubt that the vagi are the motor nerves to the forestomachs. These investigators found that cutting either the right or left cervical vagus nerve would not noticeably affect the motility of the forestomach. Cutting both of the vagi would cause an immediate paralysis of the forestomach

TABLE 1—Data Collected on 6 Sheep Randomly Selected

Sheep	No. of observations n	Average time in seconds $\bar{x}$	Deviation in seconds s_x
1	373	47.6	10.2
2	214	53.5	10.3
3	266	47.4	9.2
4	116	56.5	13.0
5	361	48.7	5.9
6	311	63.0	11.7

$\hat{m} = \frac{\bar{x}}{6} = 52.8$; $s_{\bar{x}} = 6.2$; $s_{\hat{m}} = 2.5$.

Data for 95 % confidence interval for m: 52.8 ± 6.5 seconds

n	$\hat{m}$	$s_{\hat{m}}$	$1.96 s_{\hat{m}}$
6	52.8	2.5	6.5

Data for 95 % tolerance interval for the distribution* of sheep means with 95 % confidence: 52.8 ± 27.2 seconds

n	$\hat{m}$	$s_{\bar{x}}$	k	$k s_{\bar{x}}$
6	52.8	6.2	4.414	27.2

* Where k is obtained from table 16, p. 334, in Dixon and Massey's "An Introduction to Statistical Analysis."

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and a cessation of rumination. Stimulation of either vagi will cause a contraction of the reticulum and rumen indicating that the motor nerves to the forestomach are in the vagus. These investigators also made extensive studies of the motor function of the various branches of the vagus nerves.

In our investigations, we cut both the right and left vagal nerves in the cervical region. We found, like the above investigators, that cutting either the right or the left nerves did not seem to alter the motility of the forestomach.

Figure 1 shows some graphs made before and after cutting the left vagus nerve. From a superficial examination, it would seem that there was little if any alterations of the motility. To check this we made careful measurements of the motility of the reticulum and rumen in 1 sheep before and after vagotomy (table 2). These measurements showed that there was a statistically significant slowing of the motility of the reticulum and the first rumen contraction. The second rumen contraction slowed, but it was not statistically significant. Unfortunately, graphs made on other animals with unilateral vagotomies did not lend themselves to accurate measurement. Hence, we have only the data on 1 sheep. This point may prove to be significant if more animals are investigated.

TABLE 2—Measurements of the Motility of the Reticulum and Rumen in "Old Gal"—Before, Ten Minutes After, and Seven Days After Left Vagotomy

	n	$\bar{x}$	s	t
First Reticular Contraction				
Before	40	2.09	.66	
				7.79*
After	50	3.04	.50	
Second Reticular Contraction				
Before	40	3.38	1.26	
				4.60*
After	50	4.29	.55	
First Rumen Contraction				
Before	40	7.31	.87	
				5.05*
After	48	9.26	2.31	
Second Rumen Contraction				
Before	36	6.66	1.39	
				0.533
After	25	6.87	1.78	

* Significant at 1 % level.

Table 2 indicates that after vagotomy the contraction times of all phases except the second rumen contraction are significantly slower.

n—number of observations; $\bar{x}$—average time in seconds for the various stomach movements; s—variation.

Discussion

The vagi are the motor nerves to the reticulum as indicated by stimulation and sectioning experiments.

In 1 sheep, we found a statistically significant slowing of the reticular and first rumen contraction when we cut the left vagus.

Decerebrate Studies

So far it has been demonstrated that the vagus nerves are the motor nerves to the reticulum and rumen. Let us now consider the parts of the brain that control the impulses passing down these nerves. We prepared 6 decerebrate preparations in order to determine if the medulla is the motor center controlling the forestomachs.

Methods. — The sheep were anesthetized with ether. An area over each parietal bone was shaved and prepared for surgery. Two flaps of skin, one over each parietal bone, were resected and laid down exposing the parietal muscles. The median portion of each of these muscles were dissected free from the parietal bone and laid laterally. Then two holes ½ in. in diameter were trephined on either side of the midline. These openings were made over the very posterior part of the cerebral cortex. The openings were joined by removing the bone between them with rongeur forceps. This had to be done with great care in order not to damage the dorsal longitudinal sinus. The cerebral hemispheres were moved forward by retractors. Hot cautery knives were used to sever the midbrain anterior to the pons. The main source of hemorrhage is from the basilar artery. We usually controlled this by pressure with a blunt instrument. In 4 experiments, the entire brain anterior to the pons was removed from the cranial cavity. In 2, it was severed and left in place.

A balloon was placed in the reticulum of 5 of these preparations through a fistula previously made in the rumen. This balloon was connected to a kymograph which would record any activity. From time to time, manual palpation of the reticulum was performed in order to determine if any activity were present. In 1 individual no fistula was made, but a stethograph was attached in order to determine if any reticulo-ruminal sounds were present.

Discussion of the Decerebrate Preparations

Respiratory System.—All of these preparations were very similar in the results obtained. The respiratory rate would alter during the surgery, but directly afterwards it would become quite even. All of

the preparations had the ability to breathe without external assistance. In 2 animals, artificial respiration was necessary for twelve and twenty minutes, after the surgery. These cases both developed Cheyne-Stokes rhythm upon cessation of artificial respiration (table 3B). This was probably due to the alterations of blood gasses by hyperventilation. The other 4 preparations did not show this phenomenon. The fact

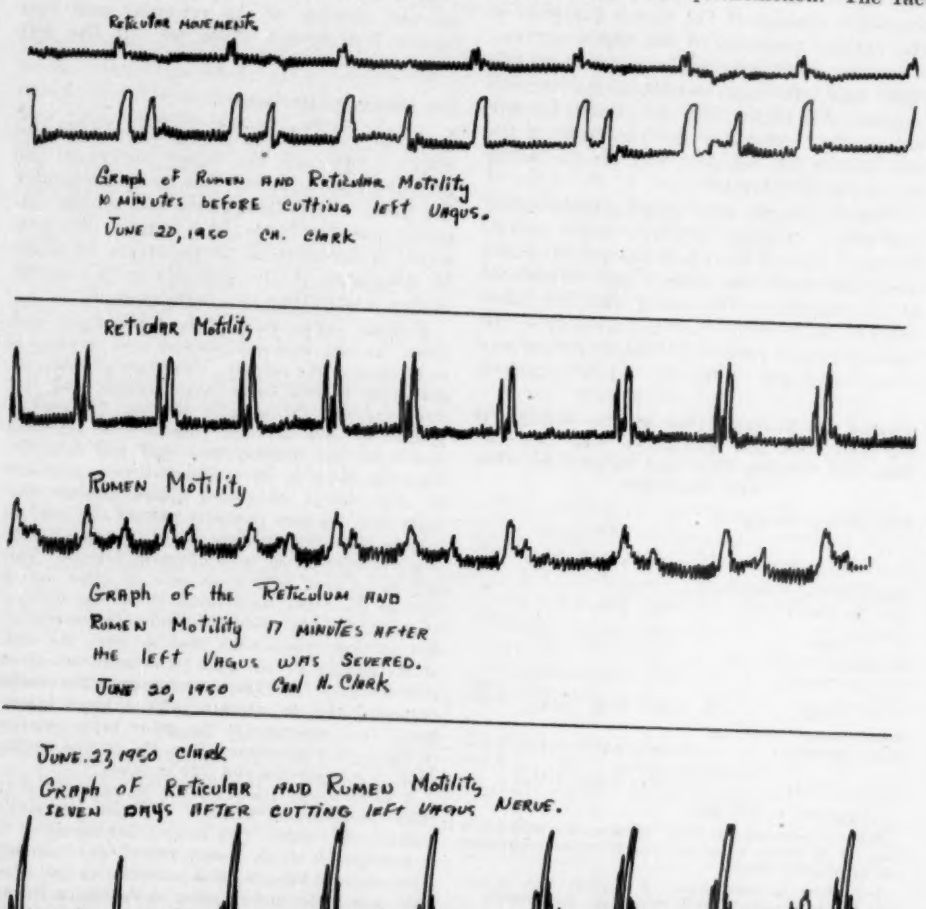
Reticular movements
Graph of Rumen and Reticular Motility
10 minutes before cutting left Vagus.
June 20, 1950 CH. Clark
Reticular Motility
Rumen Motility
Graph of the Reticulum and
Rumen Motility 17 minutes after
the left Vagus was severed.
June 20, 1950 Carl H. Clark
June. 23, 1950 Clark
Graph of Reticular and Rumen Motility
seven days after cutting left Vagus Nerve.
Rumen Contractions

Fig. 1—Motility recordings of a sheep before and after left vagotomy.

that respirations appeared normal indicates the respiratory areas of the medulla were in functioning order.

Circulatory System.—In 2 animals, the blood pressure was recorded with a mercury manometer by inserting a cannula in the femoral artery. The blood pressure was quite steady. During extensor spasms, it would rise 10 to 15 mm. of hb. pressure. After surgery, hypotension, which quite frequently characterizes spinal preparations, was not present. Apparently, the blood pressure regulating mechanism of the medulla was functioning.

Reflexes.—Stretch reflex.—The knee jerk (stretch reflex) was greatly exaggerated. Clonus was never exhibited.

Typical decerebrate rigidity was exhibited in all of the preparations. There was a severe extension of the limbs coupled with some opisthotonus. These spasms were mild at first, but they gradually became more severe during the experiment.

Heat Regulation.—As would be expected in a decerebrate preparation, these animals had little, if any, ability to regulate their body temperatures. If the wool were removed and the room cooled, then the body temperature would drop 2 or 3 degrees within four hours. On the other hand, some sheep constantly shivered even though the temperature would rise to 107 degrees. In most of the animals, the body temperature would go to 106 degrees or more. These animals developed tachycardia and polypnea. The facts indicate that temperature control, a function of the hypothalamus, was lacking in these animals.

RETICULO-RUMINAL MOTILITY AND RUMINATION

In no case was any spontaneous motility of the reticulum and rumen noted. Attempts were made to cause regurgitation by rubbing the mucosa of the rumen with roughage. These were without success. Contractions of the reticulum could always be caused by stimulating the vagi.

Spontaneous.—Swallowing movements were often noted during the various experiments indicating that the esophagus was in functioning order.

TABLE 3A—Temperature, Heart Rate, Respiratory Rate, and Motility for Sheep 1 (see Table 3B)

Time	Animal's temperature	Heart rate	Respirations per minute	Reticulum and rumen motility	Comment
12:30	103.4	72	28	None	Decerebration finished
12:45	103.4	100	43	None	Cheyne Stokes type respiration
1:30	103.0	120	96	None	Cheyne-Stokes respiration
2:00	103.0	132	70	None	Normal respiration
4:00	103.8	128	106	None	Regular respiration
6:00	103.8	105	55	None	Regular respiration
8:00	104.0	130	42	None	Regular respiration
1:00	104.2	160	37	None	Regular respiration
7:30	106.8	200 over	43	None	Regular respiration
9:00	107.1	.....	29	None	Heart rate too fast to count
9:10	107.1	.....	....	.....	Animal died

DISCUSSION

These experiments indicate that a decerebrate preparation does not have the ability to cause movements of the reticulum and rumen (table 3B). It is obvious, at least in our experiments, that there were no movements of these compartments. One criticism that might be made, however, is that the medulla was so badly damaged during the operative procedure that it was unable to cause motion of the stomach. In

TABLE 3B—A Summary of the Data Obtained in 6 Decerebrate Preparations

Animal	Length of survival	Variation in temperature	Variation of respirations	Variation in heart rate	Reticulum and rumen motility	Comment
1	20 hr. 40 min.	102.6-107.1	28-106	72-too fast to count	None	
2	6 hr. 16 min.	101.2-100.1	65-73	134-150	None	
3	9 hr.	102.0-106.0	21-64	56-148	None	Post surgical artificial respir. for 12 min.
4	17 hr. 23 min.	102.6-106.8	38-116	72-175	None	Died of tracheal obstruction
5	3 hr. 6 min.	100.6-102.8	40-58	96-140	None	Artificial respir. for 20 min.
6	23 hr. 1 min.	104.1-106.3	Not taken	Not taken	None	

order to answer this criticism, a careful examination of other known functions of the medulla was made. For example, the respiratory center in all our preparations seemed to be functioning normally. The rate, after a period of postoperative adjustment, was quite regular. In one experiment, we were able to speed up the respiratory rate by the administration of carbon dioxide gas. After the gas was withdrawn, the rate would decrease. From this evidence we felt that the part of the medulla concerned with respiration was apparently normal.

The part of the medulla concerned with decerebrate rigidity was also normal. All of our preparations showed marked decerebrate rigidity. Separating the medulla from the spinal cord always abolished this rigidity, thus demonstrating that the medulla was a necessary functioning link in the production of decerebrate rigidity.

We find that certain reflexes such as swallowing, respiration, decerebrate rigidity, and the cardiovascular reflexes were all functioning. From this it is plausible to assume that the other reflexes controlled by the medulla would also be functioning normally. Thus, if the motility of the reticulum and rumen were a function of the medulla we should have obtained contractions of these compartments in a decerebrate preparation. This assumption can neither be proved nor disproved with the evidence set forth so far.

DECORTICATE STUDIES

From the foregoing evidence, we will assume that decerebrate preparations do not have the necessary neural connections to cause motion of the reticulum and rumen. Therefore, some area of the brain anterior to the pons must be responsible for the control of forestomach motility. In order to eliminate the cortex as an area controlling reticular-ruminal motility, 10 decorticate and hemidecorticate preparations were made.

TABLE 4—The Effects of Cerebral Cortex Removal on Rumination and Eating

Sheep No.			
	Before removal of the left cerebral cortex		After removal of the cortex
CTZ	Rumination	6 hr. 52 min. / 24 hr.	5 hr. 48 min. / 24 hr.
	Eating	7 hr. 6 min. / 24 hr.	7 hr. 37 min. / 24 hr.
	Before bilateral frontal cortex removal		After frontal cortex removal
JW	Rumination	5 hr. 26 min. / 24 hr.	7 hr. 56 min. / 24 hr.
	Eating	5 hr. 30 min. / 24 hr.	6 hr. 42 min. / 24 hr.
	Before complete cortex removal		After complete cortex removal
CMP	Rumination	8 hr. 26 min. / 24 hr.	7 hr. 48 min. / 24 hr.
	Eating	8 hr. 6 min. / 24 hr.	6 hr. 48 min. / 24 hr.

The 3 sheep above were selected as typical examples from our decorticate studies. The times reported in the table are based upon 192 hours of observation. This data was collected several months before and after the operation.

From the results of these experiments we conclude that removal of the cerebral cortex has little if any effects on the amount of time spent eating and ruminating per day.

Method.—The animal was anesthetized with an ether-chloroform mixture, or pentabarbital sodium. Flaps of skin and the parietal muscle were resected and laid laterally similar to the decerebrate operations. An opening was trephined into the cranial cavity; then it was enlarged by rongeur forceps. Next the dura mater was incised and the cortex exposed. To remove the cortex we used two methods. In one method, the cortical tissue was cut free with a scalpel and the bleeding was controlled by thrombin and gel-foam sponges. This method was the more severe on the animal and produced considerable bleeding. A second method was more successful. In this technique, we cauterized the grey mater with a heated scalpel. Then the grey mater could be removed with little if any bleeding. It would not be necessary to remove the

TABLE 5—Sheep with Frontal Lobectomy

Time	Rumination per 24 hr.	Eating per 24 hr.	Resting per 24 hr.	Total hr. of observation
Before the operation	5 hr. 48 min.	7 hr. 37 min.	10 hr. 35 min.	93 hr.
One week after a frontal lobectomy	8 hr. 28 min.	8 hr. 6 min.	7 hr. 26 min.	24 hr.
Two weeks after a frontal lobectomy	11 hr. 26 min.	8 hr. 1 min.	4 hr. 33 min.	24 hr.

This sheep doubled the time spent ruminating, after a frontal lobectomy, but the food consumed and the time spent eating were not altered. This type of syndrome results after damage to subcortical areas anterior to the pituitary stalk.

cortex after killing the tissue with heat; however, it is more assuring to know that it is out and not functioning. The flaps of skin and muscle were sutured in place without trying to replace the bone.

In some of these animals, we removed the left cerebral cortex and in others the right cerebral cortex. In others we removed both cortices (fig. 2) or just the anterior half of both.

RESULTS

Vision.—The most noticeable sign of cortical removal was the impairment of vision. The opposite eye from the destroyed cortical area would be blind. For example, removal of the left cerebral cortex would cause blindness in the right eye.

Disturbances of Gait.—In some of our first animals operated, various disturbances of gait would occur on the opposite side from the lesion. However, as our operative techniques became better little if any disturbances of gait occurred. These animals lived up to a year and a half after the operation without showing any disturbances of movements.

Reticular-Ruminal Motility and Rumination.—No disturbances of reticular ruminal motility or rumination could be found in any of these animals.

In order to determine if there was any effect of cerebral cortex removal on rumination, we measured the amount of time that each of these operated animals spent eating and ruminating per day. These observations were made over at least a 48-hour period before, and a 48-hour period after, the operation.

We found that the same amount of time was spent ruminating before and after the various operations.

To measure the effect of cortical extirpations on reticulo-ruminal motility, fistulas were established in 7 of the 10 sheep. Balloons were placed in various parts of the

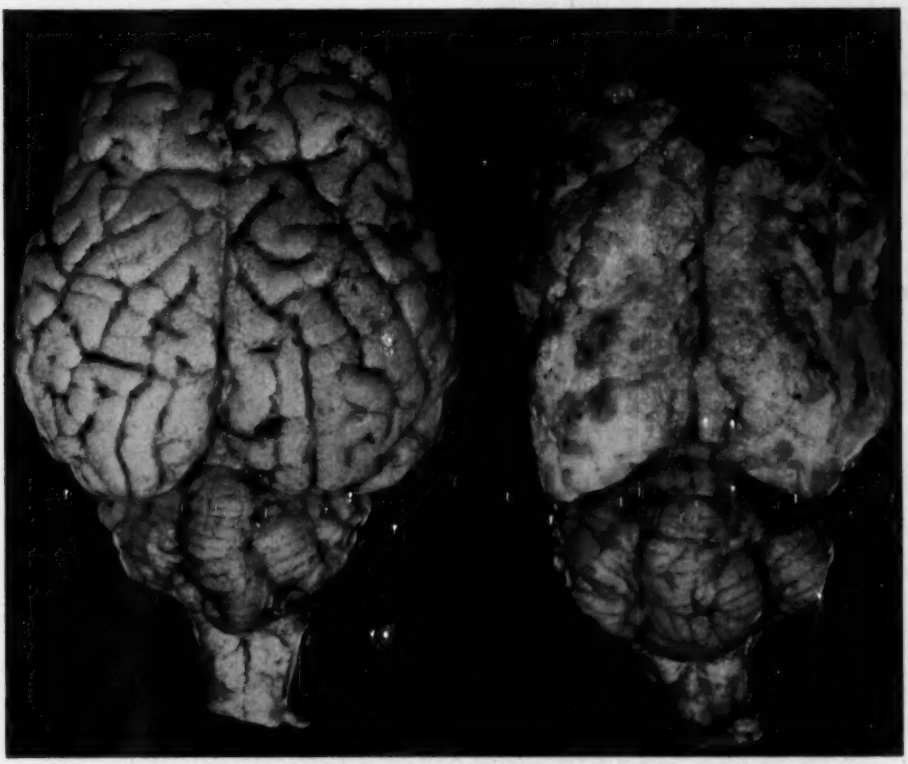

Fig. 2—The brain of sheep CMP (complete cortex removal) on autopsy as compared to the normal brain (left).

stomach and the motility recorded. The frequency and sequence of the various contractions were measured. Some of the results of the measurements are given in table 4.

From the above discussion, it is obvious that the cerebral cortex is not necessary for normal motility of the reticulum and rumen. Neither is it necessary for rumination.

FRONTAL LOBECTOMY

From the experiments on decerebrate and decorticate preparations, we will theorize that the area that controls rumination and reticulo-ruminal motility is anterior to the pons and below the cerebral cortex. In an attempt to narrow this area somewhat, 2 sheep with frontal lobectomies were prepared. The abnormalities shown in these preparations were due to the removal of subcortical tissue. This is assumed because removal of the cortex in previous experiments had no effect on rumination or reticulo-ruminal motility.

Methods.—The incision was made just slightly anterior to the optic chiasma. Both lobes were removed completely from the skull. One of the sheep was blind in the right eye apparently due to damage to the optic nerve. The other sheep had normal vision. These sheep lived sixty-three days and six months ten days, respectively, before euthanasia was performed.

On postmortem, it was found that the anterior part of the corpus callosum and caudate nucleus were removed. The hypothalamic nuclei were apparently undamaged. The examinations were made on preserved brains with a dissecting microscope.

RESULTS

Both of these animals had abnormalities in rumination. Rumen motility was apparently normal. One sheep began to spend much more time ruminating per day than it formerly had. In fact, two weeks after the operation the animal spent twice as much time ruminating per day as it formerly had (table 5). The food consumption and the amount of time spent eating per day remained about the same.

The other animal also increased the amount of time spent ruminating per day although it was not so marked—an increase average of two hours and seventeen minutes.

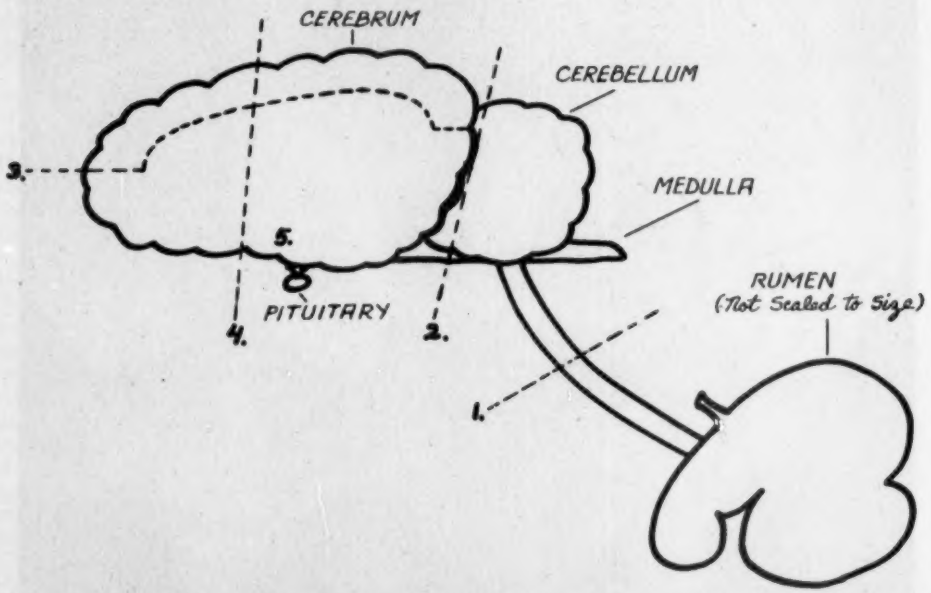

Fig. 3—Graph showing various cuts made in the central nervous system. (1) Cut vagus nerves. (2) Decerebrate preparation. (3) Decorticate preparation. (4) Frontal Lobectomy. (5) Proposed motor center for reticulo-ruminal motility.

Discussion

These effects are apparently due to injury of the subcortical tissue. Which nuclei or fiber tracts are responsible for this are not known.

Methyl Alcohol Injections

We found that making cross sections of the brain farther back than the optic chiasma caused such severe paralysis and other disturbances that the experiments were unsatisfactory. Because of this we resorted to destruction of small localized areas of the brain with methanol.

In 3 individual sheep, methyl alcohol was injected into the subcortical tissues in order to determine the effects of localized neural destruction on the brain. The injections were made with a long hypodermic needle pushed through the brain substance from above. The sight of destruction was determined on postmortem.

Results

These animals lived two days, thirty-two days, and thirty-eight days, respectively. In three successful experiments very pronounced abnormalities of rumination occurred. One animal ruminated almost continuously for 24 hours before it died. The other 2 increased the amount of time spent ruminating per day as described under frontal lobotomy. One of these lost the ability to coördinate rumination or reticulo-ruminal motility. This animal regurgitated during other parts of the cycle instead of between the two reticular contractions.

On postmortem examination, areas of liquefaction necrosis were found in the ventral part of the brain. Most of the lesions in the 2 sheep examined were anterior to the anterior commissure and ventral to the genu of the corpus callosum. One sheep had some damaged areas in its left hypothalamic nuclei. This sheep, curiously enough, was the one that lost coördination between rumination and the motility of the forestomach.

Conclusions

There seems little doubt that the vagus nerves contain the motor fibers to the reticulum and rumen. The afferent fibers are probably in the vagi too. This is based on the fact that animals with a double vagotomy do not ruminant. Also cutting the sympathetic nerves to the rumen does not hinder rumination. This gives presumptive evidence that the afferent fibers are contained in the vagi. Until their actual demonstration is made, proof will be lacking.

Next is the problem of what part of the brain controls the movements of the forestomach. Other reflexes such as vomiting, deglutition, respiration, and micturation are controlled by specific areas in the central nervous system. These together with their afferent and efferent connections control the reflexes, although they may be modified by higher centers. Many of these reflexes have been located experimentally by sectioning the central nervous system at various levels (fig. 3). Some well-known examples are the lack of the ability of decerebrate dogs to control their body temperature, because the heat regulating mechanism is in the hypothalamus. On the other hand, decerebrate preparations are able to breathe, but spinal animals* are not. This, of course, is due to the removal of the respiratory center in the medulla. Finally, spinal animals have a knee-jerk which is present until the correct part of the cord is destroyed. By these same isolating techniques, we have attempted to locate the rumination and rumen motility center.

Our decerebrate preparations never had any spontaneous motility of the reticulum and rumen. This lends evidence to the postulation that the medulla, pons, cerebellum, and spinal cord, and the various nerves attached to these structures, are not sufficient to cause rumen motility and rumination. However, we obtained normal motility with decorticate preparations. We take this to mean that the rumination center is anterior to the pons and below the cerebral cortex. By methanol injections, and surgical injury, we have obtained evidence to indicate that the rumination center may be below the genu of the corpus callosum and anterior to the pituitary stalk.

These experiments were not accurate enough to indicate the exact area. Speculation might lead us to believe that the caudate nucleus or hypothalamus might be

* The term "spinal animal," as we use it here, refers to an animal with a transection of the spinal cord immediately below the medulla. (Wiggers, C. J.: Physiology in Health and Disease, 4th ed., (1944): 143.)

very important in the rumination reflex. This, of course, will have to wait further experimentation.

SUMMARY

1) Evidence is presented to support the hypothesis that an subcortical area anterior to the pituitary infundibulum is intimately concerned with rumination and reticulo-ruminal motility.

2) Evidence has been presented to indicate that the medulla does not have the center for the control of rumination and the motility of the reticulum and rumen.

3) The cerebral cortex of either or both hemispheres is not essential for rumination or motility of the reticulum and rumen.

4) The motor nerves to the reticulum and rumen are in the vagi.

5) In 1 sheep, there was a statistically significant slowing of the motility in the reticulum and rumen after unilateral vagotomy.

References

[1] Phillipson, A. T.: Movements of the Pouches of the Stomach of Sheep, Quart. J. Exptl. Physiol., *29*, (1939): 395.

[2] Schalk, A. F., and Amadon, R. S.: The Physiology of the Ruminant Stomach, North Dakota Agric. Exper. Sta. Bull., *216*, 1928.

[3] Czepa, A., and Stigler, R.: The Roentgen Ray Picture of the Ruminant's Abdomen, Fortschr. d. naturw. forsch., *6 N.S.*, (1929): 1-78.

[4] Mangold, E., and Klein, W.: Bewegungen und Innervation des Wiederkauermagens. G. Thieme, Leipzig, 1927.

[5] Hoflund, S.: Kroniska Digestionsrubbningar hos Idisslare som Följd av Skador å N. Vagus. 5 Nordiske Veterinaermøde, Köpenhamn, (1939): 536-915.

[6] Hoflund, S.: Untersuchungen über Störungen in den Funktionen der Wiederkäuermagen, durch Schädigungen des N. Vagus verursacht. Collected papers from the Veterinary Institute, Stockholm, *14*, 1940.

[7] Sporri, Von H.: Physiologie, Pathologie und Pharmakologie der Vormägen der Wiederkäuer. Supplement April, (1951): 1-80.

Replacement Therapy with a Slowly Absorbed Progesterone Product

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Literature Cited

Previous studies[1] demonstrated the value of daily injections of crystalline progesterone in sesame oil in the maintenance of pregnancy in the cow after corpus luteum ablation. Increased interest has been shown recently in aqueous solutions of microcrystalline progesterone which form implants upon injection and therefore are slowly absorbed. Miescher *et al.*[2] as early as 1944 reported the effect of aqueous solutions of ovarian hormones to last several times as long as oil solutions in the rat. Bratt and Burch[3] reported case histories whereby single injections of "repositol" progesterone, diethylstibestrol, and testosterone resulted in a prolonged effect of the particular hormone. Murray and Eden[4] were able to inhibit the occurrence of estrus in bitches by the bi- or tri-weekly administration of 1.0 to 1.5 mg. of repositol progesterone per pound of body weight.

It is the purpose of this paper to report the use of repositol,* which is an aqueous solution of microcrystalline progesterone, as replacement therapy for extirpated corpora lutea from 5 cows in early pregnancy.

Materials and Methods

Five mature grade Holstein-Friesian cows averaging 1,200 lb. in body weight were used for this study. These cows were negative to blood serum agglutination tests for brucellosis before and after the experiment. The husbandry was excellent, the cows were kept on a hay and grain ration throughout the year, and were bred by natural service. Breeding was begun in March, 1951, and completed in May, 1951. Day one of pregnancy was considered the day following insemination. These cows had been pregnant one or more times previously.

Pregnancy was confirmed by palpation of the uterus through the rectum. Between the sixty-third and ninety-fourth day of pregnancy, each cow was subjected to surgical removal of the corpus luteum through an incision in the anterodorsal wall of the vagina.[5] In all cases, the corpus luteum was completely removed from the body cavity, and a histological section prepared therefrom to confirm complete removal. Both ovaries were examined for corpora lutea at the time of surgery.

The aqueous solution of microcrystalline progesterone used (repositol) precipitated the progesterone upon injection to form an implant. Intramuscular injections were given in the gluteal region, using a 16-gauge, 3-in. needle.

Results

The findings reported herein are a portion of a study previously reported[1] in part, in which all control animals aborted when the corpora lutea were removed in early pregnancy. Pregnancy was maintained in a high percentage of the experimental cows after corpus luteum removal, provided daily injections were given of 100 mg. of crystalline progesterone in sesame oil.

Cows 453, 466$_1$, and 454 were subjected to corpus luteum removal on the seventy-eighth, sixty-third, and ninety-fourth days, respectively, of their gestation periods (table 1). An injection of 500 mg. of repositol progesterone was given each cow on the day of surgery, and each tenth day thereafter for varying lengths of time. Periodical rectal examination of the cows indicated the fetus of each cow was developing normally. Cow 453 received the last injection of repositol on day 158 of the pregnancy. This cow did not receive exogenous progesterone again until the 249th day of pregnancy. During this interval the fetus developed normally as judged by rectal examination. After day 249 of the pregnancy, the cow was transferred to another experiment.

Cow 466$_2$ received the last injection of repositol progesterone on the 143rd day of pregnancy. The fetus developed normally as judged by rectal palpation until day 203 of pregnancy when the cow died incident to experimental surgery. Cow 454 received the last injection of repositol progesterone on the 174th day of pregnancy. No additional injections were given until the 264th

* Repositol used in this experiment was furnished by Pitman-Moore Co., Indianapolis, Ind.

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This study was conducted while the author (LEM) was a research fellow of the American Veterinary Medical Association. Present address: College of Veterinary Medicine, University of Illinois, Urbana.

day of pregnancy. The fetus developed normally as evidenced by rectal palpations, and the cow was transferred to another experiment on the 264th day of the gestation period.

The next 2 cows were studied using a more prolonged interval between injections. Cow 947_a was subjected to corpus luteum removal on the 77th day of the gestation period, and received injections of 500 mg. of repositol progesterone on days 67, 80, and 93 of the pregnancy. Resorption of the fetus of cow 947_a, as determined by rectal palpations, began soon after the corpus luteum was removed. Resorption was complete by the 106th day after breeding, and the injections of repositol progesterone were discontinued.

The corpus luteum was removed from cow 948_b* on the sixty-seventh day of pregnancy. Injections of 500 mg. of repositol progesterone were given on days 67, 80, 93, and 103 of the pregnancy. Cow 948_b aborted an intact fetus seventeen days after the last injection of repositol progesterone.

DISCUSSION

A vehicle for steroid hormones that would result in a prolonged action is very desirable in the practice of veterinary medicine. If a slow rate of release of the hormone can be effected, then the postinjection blood level fluctuation can be minimized. This would result in a lower total daily dose being required for physiological effect. Less frequent dosage is an important economic consideration.

Three of 3 cows maintained pregnancy after corpus luteum removal while receiving a dosage of microcrystalline progesterone amounting to 50 mg. or less daily when administered as a 500-mg. dose once each ten days. This compares favorably with the results obtained by the daily administration of 100 mg. of crystalline progesterone in sesame oil.[1]

One of the cows (948_b) maintained early pregnancy after corpus luteum removal while receiving a dosage of repositol progesterone amounting to 38 mg. or less daily, when administered as a 500-mg. dose once each 13 days. Resorption of the fetus of cow 947_a may indicate that the dosage was too low to simulate the output of progesterone by the functioning corpus luteum of pregnancy. The abortion of the fetus by cow 948_b further extends our observations that the corpus luteum is essential to the maintenance of early pregnancy. The results from cow 948_b may indicate that the prolonged effect of repositol progesterone in the dosage used here is not present 17 days after injection.

This report is made with the consideration that the number of experimental animals reported is too small for a statistical analysis.

SUMMARY

Three of 3 cows maintained early pregnancies after corpus luteum ablation when aqueous solutions containing 500 mg. of

* Breeding date not definite; date given based on estimate from rectal palpation at date of operation.

TABLE 1—"Repositol" Progesterone in the Maintenance of Pregnancy

Cow No.	Day of pregnancy corpus luteum removed	Therapy	Day of pregnancy therapy disc'd.	Remarks
453	78	500 mg. repositol progesterone once each 10 days.	158	Pregnancy maintained.
466_a	63	500 mg. repositol progesterone once each 10 days.	143	Pregnancy maintained until cow died on day 203.
454	94	500 mg. repositol progesterone once each 10 days.	174	Pregnancy maintained.
948_b *	67	500 mg. repositol progesterone once each 13 days, except last injection.	103	Aborted day 120.
947_a	77	500 mg. repositol progesterone once each 13 days.	Resorption began immediately after operation. Complete by day 106, therefore injections discontinued.	

* Breeding date estimated by rectal palpation.

microcrystalline progesterone were administered once each 10 days.

One of 2 cows maintained early pregnancy after corpus luteum ablation when aqueous solutions containing 500 mg. of microcrystalline progesterone were administered once each 13 days. The fetus was resorbed in the remaining cow.

"Repositol" progesterone appears to be a slowly absorbed form of progesterone, requiring less frequent and lower total dosage than is possible when an oil vehicle is used.

References

[1] McDonald, Leslie E., Nichols, R. E., and McNutt, S. H.: Studies on Corpus Luteum Ablation and Progesterone Replacement Therapy During Pregnancy in the Cow. Am. J. Vet. Res., *13*, (1952): 446-451.

[2] Miescher, Von K., Aasche, P., and Frey, H.: Depotwerkung von Kristallsuspensionen weiblicher Sexual-hormone (Ovocyclin- und Lutocyclin-Kristallampullen). Helvet. Physiol. Acta, *2*, (1944): 515-532.

[3] Bratt, H. Marvin, and Burch, George R.: A Clinical Report on Repositol Hormone Preparations. North Am. Vet., *33*, (1952): 541-546.

[4] Murray, G. H., and Eden, E. L., Jr.: Progesterone to Delay Estrum in Bitches. Vet. Med., *47*, (1952): 467-468.

[5] McDonald, Leslie E.: Vaginal Approach for Removal of the corpus Luteum, J.A.V.M.A., *120*, (1952): 128-129.

Urease and Catalase Activities of *Brucella Melitensis* from Different Geographical Regions

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A PREVIOUS study of urease in cells of the genus Brucella showed that certain strains of *Brucella melitensis* exhibited low activity of this enzyme and other strains of the species exhibited high activity.[1] Apparently, only the first group of strains was available to Pacheco and Thiago de Mello [2] who reported that strains of *Br. melitensis* exhibited low urease activity, whereas only the second group of strains was available to Bauer [3] and to Hoyer [4] who reported high urease activity. The strains in this laboratory originally occurring in the general locales of the authors mentioned above showed the same relative activities as had been reported by those authors. A geographical distribution of *Br. melitensis*, according to urease activity, was indicated.

The present study was undertaken to determine (1) whether strains showing wide differences in urease activity also show wide differences in catalase activity, and (2) whether there is a relationship between geographical distribution of strains of *Br. melitensis* and the high and low activities of the two enzymes.

EXPERIMENTAL METHODS

Cultures.—The strains of *Br. melitensis* studied were isolated from human and animal sources from 1896 to 1951. Strains which were of uncertain source or which were isolated from laboratory workers and students of veterinary medicine were not included. The comparative studies of each of the strains were made with pure smooth-intermediate (SI) colonial types, separated and identified as described by Huddleson.[5] Growth of the strains on tryptose agar plates was not inhibited by presence of basic fuchsin (1 mg./100 ml.) or by thionin (1 mg./100 ml.). There was little or no detectable sulphide gas produced by the strains when grown on liver or tryptose agar slants.

Enzymatic Measurements.—The activity of urease was measured by means of the relative rate of color change of the indicator in agar medium containing urea.[6] The urease activity of 65 per cent of the strains was also determined by a quantitative titrimetric method previously described.[1] The urease activity by the latter method is expressed as milligrams of urea decomposed by 10 x 10^9 cells in thirty minutes at 20 C.

The activity of catalase was determined by a $KMnO_4$ titration method described by Huddleson,[7] and is expressed as milliliters of 0.1 N H_2O_2 decomposed by 2 x 10^9 cells in thirty minutes.

RESULTS

Enzymatic Activities of Foreign Strains.—Fifty-three strains of *Br. melitensis* were available which were originally isolated outside the United States. The number of strains from each of the various countries, dates, and sources of isolation, and the average of results of tests for enzymatic activity on the SI types from these strains are recorded in table 1. The urease activity of 45 strains of this group, measured by rate of color change in Christensen's medium, was not more than a + reaction in one hour and ++ in four hours. The slow rate of decomposition of urea was verified by the titrimetric method; the individual strains decomposed 0.02 to 0.5 mg. urea and the average value for the group was 0.2 mg.

Eight of the 10 strains from France were found to differ from other foreign strains in rate of decomposition of urea. The color change in urea-agar was rapid and the titrimetric values of urea decomposed were 2.9 to 4.8 mg. A strain with activity similar to the French strains was from a member of the armed services hospitalized in Illinois just after returning from Italy.

The activity of catalase for each of the strains of foreign origin, including all the strains from France, was 1.8 to 24.5; the average value was 10.9 ml. of 0.1 N H_2O_2. Huddleson [7] reported the average catalase value of smooth strains of *Br. melitensis* to be 11.6.

Enzymatic Activities of Domestic Strains.—Thirty-one strains of *Br. melitensis* were studied which were isolated from sources within the United States. A group of 18 which were isolated in Iowa, Indiana, Minnesota, and Massachusetts ex-

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hibited activities of the two enzymes that were considerably greater than were noted in strains of foreign origin, with exception of the 8 from France. By the titrimetric method, this group of strains showed values for urease of 2.2 to 3.1 and values for catalase of 42.6 to 51.5.

The remaining strains isolated within the United States differed markedly from those just described in that their enzyme activities were low. The urease activity of these 12 strains was 0.15 and the catalase activity was 9.0. Five cultures of the group were isolated from students at Michigan State College.[g] Circumstantial evidence at that time indicated that the infecting strain was one which had been originally isolated in Malta. Five of the strains were isolated from sources in the southwestern part of the United States in states which border Mexico. One was isolated from a Mexican, who had been in Iowa less than a month,[f] and another from a resident of Chicago who had travelled in Mexico shortly before his acute illness.

Attempts to Alter Enzymatic Activities of Strains.—Although each of the strains of *Br. melitensis* was typical of the species with respect to growth on agar medium containing basic fuchsin or thionin in the standard concentration, some individual differences in susceptibility of strains to dyes were detected by growth inhibition determinations in higher concentrations of the dyes. On agar medium containing basic fuchsin in the concentration of 2.5 mg./100 ml., inhibition of 16 domestic strains was pronounced, although each of the strains showed slight growth. The inhibition of growth of 31 foreign strains, by basic fuchsin in this concentration, was quite varied; some strains showed no inhibition whereas others exhibited moderate, marked, or complete inhibition.

On agar medium containing thionin in the concentration of 5 mg./100 ml. the domestic strains grew as well as on medium containing the standard concentration of thionin. The foreign strains were inhibited to a moderate or marked degree by the higher concentration of thionin.

The acclimation of strains to grow in the presence of high concentrations of dyes was not paralleled by changes in urease or catalase activities or by changes in sulphide gas production. Acclimation of

TABLE 1—Urease and Catalase Activities of Strains of Brucella Melitensis

No. of strains	History of strains: Origin	Dates isolated	Source	Urease activity: mg.[a]	Urease activity: Color reactions[b] 1 hr.	Urease activity: Color reactions[b] 4 hr.	Catalase activity (ml.[c])
11	Malta	1923-1938	Human, animal	0.2	'	+;++	11.7
8	Tunisia	1921-1938	Human, animal	0.2	'	+;++	10.0
6	Turkey	1947-1948	Human	0.1	'	+;++	16.7
2	Sicily	1929	Human	0.3	'	++	6.4
2	Italy	1927-1929	Animal	0.3	+	++	9.4
2	Germany	1930	Animal	0.3	+	++	5.2
2	England	1896-1923	Human	0.3	'	++	5.6
3	Chile	.....	Human	0.1	—	+	17.7
3	Argentina	1935-1949	Human, animal	0.3	'	+;++	10.7
4	Mexico	1929-1947	Human	0.1	—	+	13.2
2	France	1930-1931	Human	0.2	+	++	7.2
8	France	1931	Human, animal	3.9	++	+++	7.5
1	Illinois[d]	1945	Human	4.0	++	+++	9.0
1	Illinois[e]	1943	Human	0.1	—	+	21.8
1	Iowa[f]	1930	Human	0.2	+	++	10.2
5	Michigan[g]	1939	Human	0.2	'	+;++	5.9
5	Southwestern U.S.	1922-1934	Human	0.1	'	+;++	9.2
18	Central and eastern U.S.	1933-1951	Human, animal	2.6	++	+++	47.2

[a] Titrimetric method; mg. urea decomposed by 10×10^9 cells in 30 min. 20 C.

[b] Christensen urea-agar; color change of indicator in agar slant: — = none, ' = surface of slant, + = slant only, ++ and +++ = penetration into butt.

[c] $KMnO_4$ titration; ml. 0.1 N H_2O_2 decomposed by 2×10^9 cells in 30 min.

[d] Patient hospitalized in Illinois on return from Italy; [e] Resident in Illinois travelled in Mexico shortly before illness; [f] Native of Mexico transient in Iowa; [g] Students at Michigan State College infected by strain of *Br. melitensis* originally isolated in Malta.

strains to a high concentration of one of the dyes did not alter ability to grow on the standard concentration of the other dye.

DISCUSSION

In a previous study from this laboratory [1] it was pointed out that certain strains of *Br. melitensis* and all strains of *Brucella abortus* showed a low urease activity whereas certain other strains of *Br. melitensis* and all strains of *Brucella suis* exhibited a relatively high urease activity. The data presented here show that strains of *Br. melitensis* also manifest differences in catalase activity. In a majority of the strains studied, the low or high activities of the two enzymes were parallel. The strains may be placed in three groups on the basis of enzymatic activities: strains from the central and eastern United States showed high activities of urease and catalase, those from France showed either high or low activity of urease and low activity of catalase, and those from other regions of the world showed low activities of urease and catalase. Strains in the last group include those isolated many years ago as well as those in recent years. They are from widely separated geographical areas in some of which the incidence of infection by this species is high. The strains isolated in California, Arizona, and New Mexico show the characteristics of the last group. On the basis of the similarity of strains from these states to strains from Mexico, the proximity of the areas and the high incidence of *Br. melitensis* in Mexico, the strains from the southwestern section of the United States are classified as Mexican strains.

Before 1943, the isolation of *Br. melitensis* from animals or man in the central section of the United States was unusual. In 1930, Jordan and Borts [9] isolated *Br. melitensis,* as well as *Br. abortus,* from one man in Iowa. He was a native of Mexico. The low activities of the two enzymes in this strain of *Br. melitensis* indicate Mexican origin. In 1943, Jordan and Borts began to isolate more strains of *Br. melitensis* from human beings in Iowa. From 1943 to 1946, 26 different isolations were made. Histories of most of the patients indicated that the probable source of infection was swine. Later, Borts *et al.*[10] isolated *Br. melitensis* from the tissues of several naturally infected swine in Iowa. Although this was the first reported isolation of *Br. melitensis* from hogs, a strain of this species was isolated from the vertebrae of a hog in Minnesota in 1933 by Dr. W. H. Feldman. It was identified as to species in this laboratory in August 1933. Studies of this strain show that its enzymatic behavior is the same as those strains isolated in Iowa and Indiana in recent years. It is evident that this variety of *Br. melitensis* was present in hogs in the central section of the United States in 1933.

Prior to 1951, the occurrence of *Br. melitensis* infection in the northern and eastern parts of the United States was confined to laboratory workers. In that year, this laboratory received a culture of Brucella isolated from an aborted swine fetus in Massachusetts. It was identified as *Br. melitensis.* The urease and catalase activities of this strain were found to be identical with those originating from sources in the central states.

Several strains of *Br. melitensis* that exhibit low activities of urease and catalase have been isolated from human beings in the central part of the United States but in each instance the sources of the infections were traced to a foreign geographical area. Thus, no strain of low enzyme activities was found which unquestionably originated in the eastern or central states.

The high enzymatic activities of strains of *Br. melitensis* occurring in the central part of the United States may be significant epidemiologically and episoötically. Sufficient information is not available to determine the extent of its spread in the United States. No clue has yet been found as to the origin of this variety. The enzymatic activities of the domestic group of *Br. melitensis* are similar to those of *Br. suis.* There is yet no positive evidence that this group is a variant of *Br. suis.* However, a noticeable inhibition of domestic strains of *Br. melitensis* by high concentration of basic fuchsin, their tolerance of high concentration of thionin, and inhibition of foreign strains by high concentration of thionin suggest relationships of the two groups of *Br. melitensis* to the other two species of the genus which are of genetic interest. Attempts to acclimate strains to grow on agar mediums containing high concentrations of basic fuchsin or thionin

were successful. Such acclimations resulted in no changes which were suggestive of a transition of any strain of *Br. melitensis* to one of the other two species of Brucella.

The strains of *Br. melitensis* studied contained one or more SI types, on the basis of colonial morphology. There were no variations in activity noted in different SI types of the same strain. Often the SI type in one strain differed in colonial appearance from that in another strain. No evidence was obtained in this study which indicated that high or low enzymatic activity was associated with a particular SI type.

The majority of the strains isolated in France could not be placed with either of the other two groups of *Br. melitensis*. Two of the French strains exhibited low enzymatic activities comparable to those of other foreign strains; eight strains showed high urease activity but low catalase activity. Renoux and Quatrefages [11] also reported a wide range of urease activities of *Br. melitensis;* however, their strains of low activity were reported as atypical *Br. melitensis* on the basis of production of H_2S. The French strains studied by the authors were isolated from sheep, goats, or human beings in the southeastern part of France within a period of two years, 1930 and 1931. Previous examinations of strains of *Br. melitensis* from that area have shown discrepancies between the H_2S and dye tests and serological tests.[12,13]

The high activities of the enzymes urease and catalase may not alone be sufficient to establish a variety of the species *Br. melitensis*. The fact that strains of these characteristics have as yet only been isolated in a limited geographical area in the United States adds to the importance of determining the activity of the two enzymes in all strains that are isolated from various sources. These determinations should furnish information as to the extent of their distribution and implantation in a new area. A comparison of virulence and a study of the dissociation patterns of the domestic, foreign, and French strains of *Br. melitensis* may elucidate the relationship of strains within that species and the relationship of strains to the species *Br. abortus* and *Br. suis*.

SUMMARY

The activities of urease and catalase in cells of smooth-intermediate colonial types of 84 strains of *Brucella melitensis* have been determined and compared. The strains showed wide differences in both urease and catalase activities. The high or low activities of each of the enzymes was characteristic of strains from certain limited geographical regions: (1) central and eastern United States—urease, 2.6; catalase, 47.2; (2) France (8 of 10 strains examined) urease, 3.9; catalase, 7.5; (3) Mediterranean Islands, North Africa, Turkey, Italy, Europe, South America, Mexico, and southwestern United States—urease, 0.2; catalase, 10.9.

Strains of *Br. melitensis* showing high activities of both enzymes, urease, and catalase, were isolated only in the central and eastern sections of the United States where infections by *Br. melitensis* had not been detected until recent years.

References

[1] Sanders, E., and Warner, J.: A Study of Urease Activity in Cells of the Genus Brucella. J. Bact., *62*, (1951): 591-598.

[2] Pacheco, G., and Thiago de Mello, M.: A Urease Test for the Differentiation of *Brucella suis*. J. Bact., *59*, (1950): 689-691.

[3] Bauer, H.: A Study of Brucella and Proteus Urease. Doctor of Philosophy Thesis. University of Minnesota, 1949.

[4] Hoyer, B. H.: Some Aspects of the Physiology of Brucella Organism in Brucellosis. Am. A. Adv. Sci., Washington, D. C., 1950.

[5] Huddleson, I. F.: Studies in Brucellosis. III. The Dissociation Pattern in the Species of the Genus Brucella. Agric. Exper. Sta., Michigan State College, Memoir 6, Part 1, (1952): 7-63.

[6] Christensen, W. B.: Urea Decomposition as a Means of Differentiating Proteus and Paracolon Cultures from Each Other and from Salmonella and Shigella Types. J. Bact., *52*, (1946): 461-466.

[7] Huddleson, I. F.: Brucellosis in Man and Animals. The Commonwealth Fund, New York, 1943.

[8] Huddleson, I. F., and Munger, M.: A Study of an Epidemic of Brucellosis Due to *Brucella Melitensis*. Am. J. Pub. Health, *30*, (1940): 944-954.

[9] Jordan, C. F., and Borts, I. H.: Occurrence of *Brucella melitensis* in Iowa. J. Am. M. A., *130*, (1946): 72-75.

[10] Borts, I. H., McNutt, S. H., and Jordan, C. F.: *Brucella melitensis* Isolated from Swine Tissues in Iowa. J. Am. M. A., *130*, (1946): 966.

[11] Renoux, G., and Quatrefages, H.: L'Indentification des Brucella par Leur Activité Ureasique. Comparaison avec Autres Methodes de Différenciation. Ann. Inst. Pasteur, *80*, (1951): 182-188.

[12] Taylor, R. M., Lisbonne, M., and Roman, G.: Recherches sur l'Identification des Brucella Isolées en France par l'Action Bactériostatique des Matiéres Colorantes et la Production d'Hydrogéne Sulfuré. Ann. Inst. Pasteur, *49*, (1932): 284-302.

[13] Wilson, G. S.: The Classification of the Brucella Group: A Systematic Study. J. Hyg., *33*, (1933): 516-541.

Preparation of Antigen for Detection of Agglutinins for *Vibrio Fetus*

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Literature Cited

The agglutination reaction has been used to some extent in the study of *Vibrio fetus* by nearly all of those who have published reports of work with this organism, starting with the original work of McFadyean and Stockman [10] on virbionic abortion of sheep in 1913. McFadyean and Stockman said nothing about the preparation of antigen except to remark on the difficulty of obtaining sufficiently luxuriant growth of the Vibrio. Smith and Taylor,[18] working with bovine strains in 1919, prepared antigen by making a saline suspension of a four-day growth on agar slants. Welch and Marsh,[20] in 1924, reported the use of saline suspensions of growth on agar slants. Knüth and David [7] spoke of the inadequate growth of the organisms for satisfactory antigen preparation but did not describe their method. Buxton [3] reported low titer agglutinins in infected sheep but did not discuss the antigen. Ryff [17] reported using the agglutination test on sheep serums but gave no information about the antigen. In England, several workers have recently reported agglutination tests with sheep serum. Brocklehurst [2] used a saline suspension of the growth on agar slants. Blakemore and Glechill [1] reported on agglutination tests in which the antigen used was a saline suspension of cells which had been grown in serum broth. Levi [8] reported the results of testing sheep serums for agglutinins, using as antigen saline suspensions of the Vibrio grown on liver agar.

In a series of publications, Plastridge has reported considerable work with agglutination antigens prepared from bovine strains of *V. fetus*. In 1941 [11] and 1943 [12] he described antigens prepared from cultures in semisolid agar mediums, which necessitated a somewhat cumbersome method of preparation. In 1949 [14] he described an antigen prepared from cultures on the surface of a solid medium made by adding agar to Difco thiol medium by a method which required redistribution of inoculum for both seed cultures and final cultures. In 1950,[15] a modification of this method was reported. The seed cultures are grown in thiol medium, and the cultures for antigen production are grown on the surface of a solid medium which is made by adding agar and glutathione to thiol medium. Here again the method requires opening the culture flask and redistributing the inoculum.

Hansen, Price, and Clements [5] have recently reported the use of a thioglycolate 3 per cent agar for the cultivation of *V. fetus* for antigen production. This work was presumably done with bovine strains.

Gallut,[4] working with strains of human and animal origin, has developed a satisfactory medium for antigen production which eliminates both agar and serum, because of the possible antigenic effect of these materials. He uses "bouillon ordinaire" to which is added 12 per cent of "extrait globulaire," which is a saline extract of red blood cells. The cultures are made in this liquid medium distributed in bottles laid horizontally so that the depth of the medium is only a few millimeters.

At the Montana Veterinary Research Laboratory agglutination antigens prepared from ovine strains of *V. fetus* have been used quite extensively during the past twenty years in connection with investigations on vibrionic abortion in sheep. The antigens used have been basically saline suspensions of the cells grown on the surface of agar mediums. There has been little difficulty in obtaining satisfactory growth for antigen production. Various modifications of the medium and the preparation of the suspensions have been tried, and comparisons with mediums and processing methods recommended by others have been made. In consideration of the difficulties reported by others and the lack of uniformity of techniques recommended, it seems useful to report the results of the work done at this laboratory.

Cultivation of Vibrio Fetus for Antigen Production

Cultivation on the Surface of an Agar Medium.—The basic medium which has been used for *V. fetus* antigen production has been a slight modification of Huntoon's "hormone" agar which, in the form of broth rather than agar, is the standard basic medium used for routine aërobic bacteriological cultures in this laboratory. To make the broth, 500 Gm. of finely ground lean beef or beef heart and 10 Gm. of bacto-peptone are added to 1000 ml. of water. The mixture is heated to 68 C., the pH is adjusted to 7.6, and two eggs, broken and stirred into a little water, are added and thoroughly mixed with the meat. The mixture is then heated in flowing steam for one hour. A firm clot forms which is separated from the walls of the container and the mixture is again steamed for one hour. The clear fluid is then poured off without filtration. For use in antigen production, bacto-agar in the amount of 1.5 per cent of the broth is added and the reaction adjusted to pH 7.8. After heating to dissolve the agar, any precipitate is allowed to settle, and the clear supernatant fluid

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is drawn off and autoclaved. The final pH is about 7.4. Before use, 10.0 per cent of sterile horse serum is added, and the medium is distributed in 50-ml. quantities in 16-oz. prescription bottles laid on the side.

It will be noted that no sodium chloride is used in the preparation of this medium. Comparison of 5 strains of *V. fetus* on salt-free medium and on medium containing 0.5 per cent sodium chloride showed that the medium containing salt had a tendency to produce pleomorphism and filamentous forms, as compared with the more consistent production of short spirilla on the salt-free medium.

The addition of horse serum to the "hormone" medium improves the growth of the organism, although a medium containing serum is often considered to be unsuitable for the cultivation of organisms for use in preparing antigen. In this case, the addition of serum to the medium has not interfered with the use of the cultures for agglutination antigens.

The culture medium is inoculated by pipetting 1 ml. of a 72-hour serum-broth culture of *V. fetus* to the surface of the medium. Approximately 15 per cent of the air in the culture bottle is replaced with carbon dioxide gas, and the bottle is plugged with a sterile rubber stopper. The cultures are then incubated for forty-eight hours at 37 C.

Several modifications of this medium were tried, with the objectives of increasing the quantity of growth, improving the quality of the antigen, and simplifying the procedure. It was found that a yeast autolysate, sterilized by filtration rather than heat, could be substituted for the serum without reducing the amount of growth, and that when yeast autolysate was used, the use of carbon dioxide gas was unnecessary for obtaining the maximum growth. But comparison of cultures grown with and without increasing the carbon dioxide in the atmosphere showed that the additional carbon dioxide was necessary to maintain the normal short spirillar form and motility of the organism which are desired for the production of a good agglutination antigen.

The medium described has been compared with the thiol medium used by Plastridge, and with the thioglycolate agar used by Hansen, Price, and Clements. In our experience there was no advantage in these mediums from the standpoint of antigen production, and the medium described here has the advantage of producing a larger amount of growth in a shorter time.

Cultivation in Liquid Medium.—The medium and conditions of incubation described above have been found generally very satisfactory for the production of antigen. However, with some strains of *V. fetus*, there has been difficulty in producing a smooth antigen in which the cells would remain in suspension. It is also found that these strains usually lose their motility during the period of growth on the agar medium, and also have a tendency to lose their spirillar morphology and change to the coccoid form.

It has been observed that *V. fetus* retains its normal morphology and its motility for a longer period in fluid medium, but it has been difficult to obtain the necessary density of growth in broth. It has now been found that "hormone" broth plus 10 per cent horse serum, distributed in 50-ml. quantities in 16-oz. prescription bottles laid on their sides so that the depth of the medium is only about 6 mm., will support a satisfactory growth of the organism when 15 per cent of the air in the bottles is replaced by CO_2 gas. A comparison of the growth of three strains grown with and without added CO_2 showed a much heavier yield in the cultures grown under CO_2. A number of experiments comparing the serum broth antigens with the serum agar antigens have shown that there are several advantages in the broth method of cultivation. The yield of cells is only slightly less from 50 ml. of broth with a depth of medium of about 6 mm. than from 50 ml. of agar. The motility is maintained more completely up to the time of harvest, and there is less change in morphology to the coccoid form. These comparisons also indicate that the antigens prepared from the broth cultures have less tendency to settle out rapidly, and are less apt to show agglutination in negative serums.

The maximum growth in this medium may occur in twenty-four hours, and the time of incubation should never exceed forty-eight hours. The cultures should be examined at twenty-four hours, when an experienced technician can judge by the density whether the maximum growth has been reached. If it appears that this point has been reached, the cultures should be immediately harvested. This is based on comparisons of the motility and morphology of the organisms in 24-hour and 48-hour cultures which show that while motility in broth cultures is well maintained through the 48-hour growth period, there is some change in morphology to the coccoid form.

The serum broth cultivation of *V. fetus*, as described above, was compared with the medium of Gallut. Gallut considered that the advantages of his "extrait globulaire" medium were in the fact that it contained neither agar nor serum proteins, and that the organism did not go into the coccoid form in that medium. A quantity of "extrait globulaire" was prepared in our laboratory and the medium described by Gallut was compared with our medium. The growth obtained in Gallut's medium was similar to that in our serum broth, except that the quantity of growth in forty-eight hours was slightly less than in the medium used at this laboratory. The antigen prepared by Gallut's method gave agglutination titers with immune serums which were practically identical with the titers obtained with antigens prepared by our method.

The effect of horse serum on the yield and sensitivity of the antigen was determined for three strains of the organism. In this trial, the yield was definitely better for two of the strains in

broth to which 10 per cent of serum had been added than in the plain broth. An agglutination test comparing an antigen prepared from a serum-broth culture and one from the same strain prepared from a plain broth culture showed no significant difference in sensitivity, as indicated by the titer of an immune serum and by the absence of any agglutination in a composite negative serum.

PREPARATION OF THE BACTERIAL SUSPENSION

The methods of preparation of the suspensions of the organisms are as follows, depending on whether the cultures were on agar or in broth.

In the case of the agar cultures, at the end of the incubation period the growth is washed off in 50 ml. of 0.85 per cent sodium chloride solution to which 0.3 per cent formalin has been added. The cells are thrown down by centrifugation and washed once in formolized saline. They are then resuspended in formolized saline, and the turbidity is adjusted photoelectrically to a point between tubes 1 and 2 of the McFarland scale.

In the case of the broth cultures, enough 3.0 per cent formalin solution is added to each bottle, after twenty-four to forty-eight hours of incubation, to make an 0.3 per cent formalin solution. The cells are thrown down by centrifugation, and washed once in 0.3 per cent formolized saline, resuspended, and the turbidity adjusted as for the suspensions from agar cultures.

SELECTION OF STRAINS OF VIBRIO FETUS FOR ANTIGEN PRODUCTION

The production of a satisfactory antigen requires not only effective methods of cultivation and processing but also that the cultures used be the smooth type. At this laboratory the form considered to be normal or smooth and satisfactory for antigen production, is the short, spirillar, actively motile form, the majority of the organisms showing one or two complete wave-lengths. Some strains have a tendency to develop long spirilla or filaments on artificial cultivation; others may rapidly change to a coccoid form. Neither of these forms is desirable for antigen production. In making an antigen for diagnostic purposes, it must also be recognized that there are several distinct serological types of *V. fetus*, as reported by Marsh and Firehammer.[9] Preliminary trials indicate that it may be possible to prepare a polyvalent antigen for diagnostic work.

The stock cultures of *V. fetus* are carried in the lyophilized state. When recovered from lyophilization, the first transfers are to serum agar slants. As soon as a luxuriant growth is obtained on the agar, the cultures are transferred to serum broth for use as seed cultures in the production of antigen. It has been found that some strains show greatly reduced motility after cultivation on the surface of agar, and that in general the motility in surface cultures declines rapidly after the first twenty-four hours. When strains which have lost motility after cultivation on agar are transferred to serum broth they become actively motile in the broth and retain their motility in broth cultures for several days. The work of Blakemore and Gledhill indicates that both flagellar and somatic antigens are involved in the production of *V. fetus* agglutinins, and their preliminary experiments indicate that there may be more strain specificity in the somatic than in the flagellar antigens. Therefore, the flagellar antigens may be of value in diagnostic agglutinations, and motile strains should be used in making antigens.

SUMMARY

A simple and effective method of preparing antigen for the detection of agglutinins for *Vibrio fetus* is described.

The basic medium for cultivation of the organism is a modification of Huntoon's "hormone" medium. The medium may be used either as an agar for surface growth or as a liquid medium. In either case, 10 per cent of horse serum is added and the CO_2 concentration in the atmosphere over the medium is increased to 15 per cent. The preferred medium is the liquid form.

In the preparation of the suspension, the cells are washed once in 0.85 per cent salt solution to which 0.3 per cent formalin has been added, and then suspended in formolized saline.

For antigen production, strains should be selected in which the short spirillar forms and motility can be maintained. In selecting strains, the serological type must also be considered.

References

[1] Blakemore, F., and Gledhill, A. W.: Studies on Vibrionic Infection of Sheep. I. The Agglutination Test as a Means of Diagnosis. J. Comp. Path. and Therap., *56*, (1946): 69-74.

[2] Brocklehurst, I. W.: *Vibrio Fetus* Agglutinins in Bovine Sera. Vet. J., *102*, (1946): 279-282.

[3] Buxton, J. B.: A Note on *Vibrio Fetus* in the Ram. First Report. Inst. Anim. Path., Univ. of Cambridge, (1929-30): 47-51.

[4] Gallut, J.: Recherches Immunochimiques sur *Vibrio foetus*. 1.—Étude Sérologique Préliminaire de 10 Souches d'Origine Diverses. Ann. Inst. Pasteur, *83*, (1952): 449-454.

[5] Hansen, P. Arne, Price, K. E., and Clements, Mary F.: Suitable Thioglycolate Media for the Cultivation of *Vibrio Fetus*. J. Bact., *64*, (1952): 772.

[6] Huntoon, F. M.: "Hormone" Medium. A Simple Medium Employable as a Substitute for Serum Medium. J. Infect. Dis., *23*, (1918): 169-172.

[7] Knuth, P., and David, Walter: Beobactungan und Utersuchungen über Spirillen als Ursache ansteckenden Verwerfens beim Rind und Schaf. Ztschr. f. Infektionskr., *33*, (1928): 19-33.

[8] Levi, M. L.: The Agglutination Test in Vibrionic Abortion in Sheep. J. Comp. Path. and Therap., *60*, (1950): 65-70.

[9] Marsh, H., and Firehammer, B. D.: Serological Types of *Vibrio Fetus*. Am. J. Vet Res., 14, (1953): 396-398.

[10] McFadyean, J., and Stockman, Stewart: Report of the Departmental Committee Appointed by the Board of Agriculture and Fisheries to Inquire into Epizoötic Abortion, Appendix to part III, Abortion in Sheep, 1913.

[11] Plastridge, W. N.: Cultural and Serological Observations on *Vibrio Fetus*. J. Bact., *42*, (1941): 816-817.

[12] Plastridge, W. N.: Observations on *Vibrio Fetus* Infection in Cattle. J.A.V.M.A., *102*, (1943): 89-95.

[13] Plastridge, W. N., Williams, L. F., and Petrie, D.: Vibrionic Abortion in Cattle. Am. J. Vet. Res., *8*, (1947): 178-183.

[14] Plastridge, W. N., Williams, L. F., and Roman, J.: An Improved Method for the Preparation of *Vibrio Fetus* Agglutination Antigen. J. Bact., *57*, (1949): 657-658.

[15] Plastridge, W. N., Williams, L. F.: Procedure for Conducting the Tube Agglutination Test for Vibriosis. Third rev. INF-29, Storrs Agric. Exper. Stat., 1950.

[16] Plastridge, W. N., Williams, L. F., Easterbrook, H. L., Walker, E. C., and Beccia, R. N.: Vibriosis in Cattle. Bulletin 281, Storrs Agric. Exper. Stat., 1951.

[17] Ryff, J. F.: Vibrionic Abortion in Michigan Sheep. J.A.V.M.A., *97*, (1940): 452-453.

[18] Smith, Theobald, and Taylor, M. S.: Some Morphological and Biological Characters of the Spirilla (*V. Fetus*, n. sp.) Associated with Disease of the Fetal Membranes in Cattle. J. Experti. Med., *30*, (1919): 299-311.

[19] Welch, H., and Marsh, H.: Vibrionic Abortion in Sheep. J.A.V.M.A., *65*, (1924): 203-210.

Serological Relationships of Twenty-Three Ovine and Three Bovine Strains of *Vibrio Fetus*

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Literature Cited

Agglutinins were demonstrated in the blood of ewes infected with *Vibrio fetus* by McFadyean and Stockman[5] in their original report on vibrionic abortion in sheep in 1913. Since then, others who have worked with this disease in sheep have reported the use of the agglutination reaction,[1, 2, 6-8, 11, 14] but only two have mentioned antigenic differences between strains of the organism. Blakemore and Gledhill compared four strains isolated from sheep, and they found three types of somatic antigens. They also found that three strains which showed no cross agglutination of the O antigen did show cross agglutination of the H antigens. Levi made agglutination antigens from five strains of *V. fetus* isolated from five outbreaks of sheep abortion. Rabbits were immunized against the five strains and cross-agglutination tests made with these serums indicated partial antigenic relationship between these strains but did not establish well-differentiated types.

In 1919, Smith,[12] and Smith and Taylor[13] reported on agglutinins produced by strains of *V. fetus* of bovine origin. They tested antigens prepared from strains isolated from 22 aborted calves against the serums of rabbits immunized against three of the strains and concluded that the strains were alike, serologically. As all the strains tested originated in one herd of cattle, this conclusion could not be interpreted as indicating that all bovine strains of *V. fetus* are antigenically alike.

Since 1941, Plastridge has published several articles dealing with the agglutination reaction of cattle serums. He does not report any evidence as to relationships in antigenic structure between bovine strains but states in his most recent publication (1951)[10] that "it appears that *V. fetus* cultures of bovine origin, unlike those of ovine origin, belong to one serologic type."

Brocklehurst[2] reported agglutination tests with a *V. fetus* antigen of bovine serums submitted for routine Brucella tests. He stated that his "antigen was monovalent, because as far as we knew at the time, *V. fetus* seemed to be an antigenically homogeneous species."

Gallut[4] has recently reported the results of cross-agglutination tests using ten strains of *V. fetus*, four of which were of human origin, and six of animal origin, including at least one ovine strain. He found ten antigenic types in the ten strains studied, with no relation to the source of the strains. He also found six antigenic factors involved, in various combinations.

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Experimental Procedure

At the Montana Veterinary Research Laboratory, the agglutination reaction has been used for many years in the study of vibrionic abortion in sheep. Early in the work with *V. fetus* it was recognized that several serological types were being found. In the past twenty years, *V. fetus* has been recovered in culture from 62 cases of vibriosis in sheep, from 34 widely distributed localities; and four ovine strains received from other workers have been studied. *Vibrio fetus* has never been recovered from cattle at this laboratory, but serological work has been done with three bovine strains received from other laboratories. Of the total 66 ovine strains, antigens have been prepared from 23, and rabbits have been immunized against 14 strains. Antigens and immune serums have been prepared from the three bovine strains which have been available.

Antigens.—The antigens used have been suspensions in 0.85 per cent salt solution of organisms grown on the surface of serum "hormone" agar, in an atmosphere containing 10 to 15 per cent CO_2. The agar is a slight modification of the medium described by Huntoon[5] as "hormone" medium which, in the form of broth rather than agar, is the basic medium used for aërobic bacterial cultures in this laboratory. For antigen production, an agar concentration of 1.5 per cent was used and 10 per cent of horse serum was added. The inoculum used was a 72-hour serum-broth culture which had been incubated in an atmosphere containing 15 per cent carbon dioxide. The antigen cultures were incubated at 37 C. for forty-eight hours. In the earlier work included in this report, the growth was washed off in 0.85 per cent salt solution, the suspension killed by heat, centrifuged, and the cells then suspended in phenolized saline. In the more recent work, the heating has been eliminated, and formalin, 0.3 per cent, has replaced phenol as the preserving agent. The growth is washed off the agar in formolized saline and diluted, without washing, to a turbidity adjusted photoelectrically to a point between 1 and 2 on the McFarland scale.

Immune Serums.—The immune serums used in the cross-agglutination tests have been prepared by injecting suspensions of living organisms intravenously into rabbits, usually using five to seven increasing doses at intervals of five days. The titers obtained with the homologous antigens have

in most cases been as high as 1: 3200 but a few were lower.

Agglutination Test Method.—The initial dilution used in the tests was 1: 25, made by adding 0.1 ml. of the serum to 2.4 ml. of the antigen. One milliliter of antigen was placed in each of the remaining tubes in the series and serial dilutions were made by transferring 1 ml. from each tube to the next tube in the series, resulting in dilutions of 1: 25, 1: 50, 1: 100, 1: 200, etc. The tests were held at 37 C. for sixteen to twenty hours. The reading was made after slight agitation of the tube, which must be done very gently as the clumps are easily broken.

The serums of rabbits which had not been immunized against the antigens used produced no agglutination in a dilution of 1: 25.

RESULTS

Six of the 23 ovine strains which have been studied serologically were worked with in the early period of the investigation and were not available to compare with the larger number of strains isolated since 1941. Rabbits were immunized against these six strains, and cross-agglutination tests were made. The results showed two distinct types, with no cross agglutination between the types. In one group, there were four strains from three widely separated localities in Montana. In the second group, there was one Montana strain and a strain isolated from a sheep in Minnesota.

The remaining 17 sheep strains, and three cattle strains which were compared serologically, were isolated between 1941 and 1952. This group was not compared with the strains of the earlier group. Antigens were prepared from all these strains, and rabbits were immunized against seven ovine and three bovine strains. Table 1 shows, in a simplified form, the results of cross-agglutination tests with these antigens and serums. The number of ovine strains shown in the table has been reduced to 14, as four of the 17 studied were from the same source.

There appear to be five serological types. Eight of the 14 ovine strains fall into type 1. Two of these eight type-1 strains originated from two outbreaks occurring spontaneously in the veterinary laboratory flock, ten years apart, and one from an ewe in the Montana experiment station band. The other five type-1 strains were from five widely separated localities in Montana and Idaho.

The type-2 group consists of one strain from the range band of the Montana Experiment Station, and a strain of ovine origin received from the BAI Animal Disease Station at Beltsville, Md., which was originally isolated from a sheep in California.

The type-3 group is composed of three strains of bovine origin, two of which were received from the Animal Disease Station at Beltsville, and one from the New York State Veterinary College. Type 3 is evidently closely related to type 2, as the

TABLE 1—Cross Agglutinations of 17 Ovine and Bovine Strains of Vibrio Fetus

Antigens				Antiserums								
				1		2		3			4	5
Type	Strain	Host	Source	2440	1828	2221	4440	2315	2857	4404	1806	4532
1	2440	Sheep	Montana Vet. Res. Lab.	++	++	−	−	−	−	−	−	−
	4847	Sheep	Montana Vet. Res. Lab.	++	++	−	−	−	−	−	−	−
	2181	Sheep	Montana State College	++	++	○	○	○	○	○	−	○
	1828	Sheep	White Sul. Spgs. Mont.	○	++	○	○	○	○	○	+	○
	4777	Sheep	McAllister, Mont.	++	+	−	−	±	−	−	−	−
	4823	Sheep	Belgrade, Mont.	++	++	−	−	−	−	−	−	−
	4858	Sheep	Weldon, Mont.	++	++	−	−	−	−	−	−	−
	4849	Sheep	Dubois, Idaho	++	++	−	−	−	−	−	−	−
2	2221	Sheep	Montana State College	−	−	++	++	+	+	+	−	−
	4440	Sheep	California	−	−	++	++	±	+	++	−	−
3	2315	Cattle	A.D.S. Beltsville, Md.	−	−	−	+	++	++	++	−	++
	2857	Cattle	A.D.S. Beltsville, Md.	−	−	−	+	±	++	++	−	++
	4404	Cattle	N. Y. State Vet. College	−	−	−	++	+	++	++	−	−
4	1806	Sheep	Montana State College	○	+	○	○	○	○	○	++	○
5	4532	Sheep	Sidney, Mont.	−	−	−	−	+	−	−	−	++
	4929	Sheep	Twin Falls, Idaho	−	−	−	+	++	++	−	−	++
	4930	Sheep	Twin Falls, Idaho	−	−	−	+	−	−	−	−	++

++—complete agglutination at 1: 100 or higher; +—no complete agglutination above 1: 25, but partial agglutination at several dilutions; ±—very slight agglutination in several dilutions; −—no more than slight agglutination at 1: 25; ○—no test.

three antiserums for the bovine type-3 strains agglutinate the two type-2 antigens to some extent.

Type 4 is represented by only one strain, 1806. A rabbit immunized against this strain agglutinated the homologous antigen to a high titer but failed to agglutinate any of the other strains used in this study, except for a low titer agglutination of strain 1828, a type-1 strain.

Type 5 is represented by three strains. This group shows some relationship to the type-3 group of bovine strains. Antiserum was prepared from only one strain of this group, 4532. This serum agglutinated all three strains of the group, and also two of the three strains of type 3. Antigen from strain 4929 was agglutinated by serum from the same two type-3 strains.

These results seem to indicate that type 1 is the predominant serological type in sheep, and that there is no antigenic relationship between this type and the other four types, except for the slight cross agglutination shown between the one type-4 strain and one strain of type 1.

The three bovine strains constitute an independent group but cross agglutination is not uniform within this group, although there is at least some slight reaction in all combinations. While our predominant ovine type shows no relation to the bovine type, the two type-2 ovine strains and two of the three type-5 strains are antigenically related to the bovine strains. In fact, one of the type-2 ovine strains, 4440, which was obtained from the Animal Disease Station at Beltsville, has been used at that station in preparation of antigen for routine testing of bovine serums.

It is recognized by the authors that this report on serological types of *V. fetus* as indicated by the agglutination reaction is incomplete in that the flagellar and somatic antigens have not been differentiated, and that further information could be obtained by using methods such as the absorption technique.

SUMMARY

The serological relationship of 23 ovine and three bovine strains of *Vibrio fetus*, as indicated by the production of agglutinins in immunized rabbits, have been investigated. The ovine strains fall into four types, while the three bovine strains are of a fifth type. The majority of the ovine strains are of one type, which is sharply differentiated from the other four types. The bovine type shows relationship to two of the less common ovine types.

References

[1] Blakemore, E., and Gledhill, A. W.: Studies on Vibronic Abortion of Sheep. II. The Antigenic Relationship between Strains of *V. Fetus*. J. Comp. Path. and Therap., *56*, (1946): 74-77.

[2] Brocklehurst, I. W.: *Vibrio Fetus* Agglutinins in Bovine Sera. Vet. J., *102*, (1946): 279-282.

[3] Buxton, J. B.: A Note on *Vibrio Fetus* in the Ram. First Rep., Inst. Anim. Path., Univ. of Cambridge, (1929-30): 47-51.

[4] Gallut, J.: Recherches Immunochimiques sur *Vibrio Fetus*. 1.—Étude Sérologique Préliminaire de 10 Souches d'Origines Diverses. Ann. Inst. Pasteur, *83*, (1952): 449-454.

[5] Huntoon, F. M.: "Hormone" Medium. A Simple Medium Employable as a Substitute for Serum Medium. J. Infect. Dis., *23*, (1918): 169-172.

[6] Knuth, P., and David, Walter: Beobachtungen and Untersuchungen über Spirillen als Ursache ansteckenden Verwerfens beim Rind und Schaf. Ztschr. f. Infektionskr., *33*, (1928): 19-33.

[7] Lee, A. M., and Scrivner, L. H.: Experimental Work Upon Recent Outbreaks of Abortion in Ewes. Am. J. Vet. Res., *2*, (1941): 50-54.

[8] Levi, M. L.: The Agglutination Test in Vibrionic Abortion in Sheep. J. Comp. Path. and Therap., *60*, (1950): 65-70.

[9] McFadyean, J., and Stockman, Stewart: Report of the Departmental Committee Appointed by the Board of Agriculture and Fisheries to Inquire into Epizoötic Abortion, Appendix to Part 3, Abortion in Sheep, 1913.

[10] Plastridge, W. N., Williams, L. F., Easterbrook, H. L., Walker, E. C., and Beccia, R. N.: Vibriosis in Cattle. Bull. 281, Storrs Agric. Exper. Sta., 1951.

[11] Ryff, J. F.: Vibrionic Abortion in Michigan Sheep. J.A.V.M.A., *97*, (1940): 452-453.

[12] Smith, Theobald: The Etiological Relation of Spirilla *(Vibrio Fetus)* to Bovine Abortion. J. Exptl. Med., *30*, (1919): 313-323.

[13] Smith, Theobald, and Taylor, M. S.: Some Morphological and Biological Characters of the Spirilla (*V. Fetus*, n. sp.) Associated with Disease of the Fetal Membranes in Cattle. J. Exptl. Med., *30*, (1919): 299-311.

[14] Welch, H., and Marsh, H.: Vibrionic Abortion in Sheep. J.A.V.M.A., *65*, (1924): 203-210.

Experimental *Vibrio Fetus* Infection in Guinea Pigs
I. Bacteriological Aspects

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THE NUMBER of reports [1,2,5,8-10,13,16] describing *Vibrio fetus* infections among cattle indicate that the infection is widespread. In addition to causing abortion, *V. fetus* has been suspected as an etiological factor of sterility.

The pathogenicity of *V. fetus* for laboratory animals has received little study. This may be ascribed in part to the earlier experiments [6,14] which indicated that infections could be produced in mice, rabbits, and guinea pigs only with difficulty, or not at all.

LITERATURE CITED

Smith [14] found *V. fetus* to be nonpathogenic for guinea pigs, rabbits, rats, and mice by the intraperitoneal route of inoculation. Pregnant guinea pigs are susceptible to *V. fetus* infection according to Lerche,[6] who succeeded in causing abortion with intraperitoneal, conjunctival, and subcutaneous inoculations. Lerch[6] did not state whether *V. fetus* was isolated from the uteri of the animals which aborted and it is probable that his results were based entirely upon microscopic examination. He was not able to demonstrate Vibrio organisms in the stomach contents of aborted fetuses. Plastridge and Williams [8] cultivated *V. fetus* in the allantoic fluid of 12-day-old embryonated hens' eggs. The embryos died within five days following inoculation. McFadyean and Stockman,[7] Smith,[15] and Simon [12] succeeded in producing abortion in cattle by the intravenous inoculation of cultures or saline suspensions of *V. fetus*. In reviewing the subject of infectious abortion in cattle, Schroeder [11] postulated that *Br. abortus* paved the way for *V. fetus* infections.

The scope of this paper is limited to the bacteriological and gross pathological aspects of experimental *V. fetus* infection in guinea pigs. In addition, the effect of concurrent *Br. abortus*, U.S.D.A. strain 19, with *V. fetus* infection in guinea pigs was investigated.

MATERIALS AND METHODS

The gravid animals selected for these experiments were estimated to be in the fourth to seventh week of pregnancy.

The material containing the Vibrio organisms was prepared for inoculation by growing the cultures in 16-oz. medicine bottles of Albimi Brucella agar which were slanted to provide the greatest possible surface area. Five grams of plain agar were added to each liter of the medium to increase its solidity. Incubation was at 37 C. in an atmosphere of 10 per cent carbon dioxide for ninety-six hours. The resultant growth was washed from the agar surface with sterile 0.85 per cent sodium chloride solution and filtered through sterile glass wool. The material for inoculation was standardized so that 0.5 ml. of this suspension added to 10 ml. of saline gave a reading of 67 per cent light transmission in the Evelyn photoelectric colorimeter with the 660 μ filter. The density approximated tube 3 of the McFarland nephelometer.

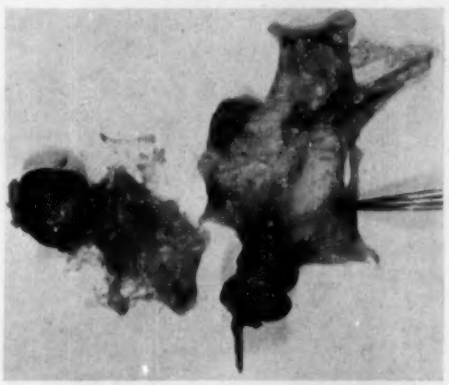

Fig. 1—Hemorrhagic, edematous, and necrotic areas of placenta and fetal membranes of a guinea pig which aborted at twelve days following intraperitoneal exposure with Vibrio fetus.

The uterine fluids and fetal stomach contents from the animals of all experimental groups were cultured in tubes of Albimi Brucella broth containing 0.1 per cent plain agar and on plates of Albimi Brucella agar for the presence of *V. fetus* and *Br. abortus*, respectively. Incubation was at 37 C. in an atmosphere of 10 per cent carbon dioxide. The cultures were incubated for one week before they were discarded and considered to be negative. The uterine fluids and fetal stomach contents also were examined by two microscopic methods, *i.e.*, slides

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stained by the Gram technique and wet mount preparations for the presence of motile Vibrio organisms. The spleens and axillary lymph nodes

Fig. 2—Uterus of guinea pig which aborted at seven days following oral exposure to Vibrio fetus. Areas of endometrical hemorrhage, necrosis, and edema are evident.

of the animals which had been inoculated with *Br. abortus*, U.S.D.A. strain 19 (experiment 1, groups B and C), were cultured for the presence of Brucella using plates of Albimi Brucella agar and a special selective Brucella medium.[4]

Experiment 1.—Thirty-five female guinea pigs were divided into four groups. Groups A and B each consisted of 7 pregnant and 3 nonpregnant animals; group C contained 6 pregnant and 4 nonpregnant guinea pigs; group D was comprised of 4 pregnant and 1 nonpregnant animal.

Group A animals received intraperitoneal inoculations of *V. fetus* suspended in a sterile 0.85 per cent sodium chloride solution on days 1, 3, 5, and 7. The inoculum consisted of 1 ml. of a mixed culture, *i.e.*, *V. fetus* strain 543 of ovine origin and strain P1 of bovine origin.

Group B received a saline suspension of approximately 10^8 cells of *Br. abortus*, U.S.D.A. strain 19, subcutaneously, in the axillary region.

Group C received the same inoculum in the same manner as did group B. One week after being inoculated with the Brucella organisms, the animals were inoculated intraperitoneally on alternate days with *V. fetus* in the same manner as employed with group A.

Group D served as a control and each animal was given 1 ml. of the sterile saline solution by the intraperitoneal route following the same regimen as groups A and C.

Experiment 2.—Ten guinea pigs, 6 of which were pregnant and 4 of which were nonpregnant, were exposed to *V. fetus* by the intravaginal route. One milliliter of a saline suspension of *V. fetus* prepared as described in experiment 1 was administered on alternate days until five inoculations had been made. Four pregnant and 1 nonpregnant animal were treated in a similar manner employing sterile saline. These animals served as controls for this experiment.

Experiment 3.—Nine pregnant and 1 nonpregnant guinea pigs were given 2 ml. of a saline suspension of *V. fetus* by the oral route daily for six consecutive days. The inoculums were cultured and prepared in the same manner as described in experiment 1, except that the organism had been subjected to five serial intraperitoneal guinea pig passages. This was done to maintain or increase any inherent pathogenicity possessed by the culture. Four pregnant animals under the same regimen were given 2 ml. of sterile saline by the oral route and served as controls. Blood serum was collected from both experimental and control animals at the time of slaughter and examined for the presence of *V. fetus*, strains 543 and P1, and *Br. abortus* agglutinins by the tube-agglutination test method.

Experiment 4.—Seven gravid and 3 nongravid guinea pigs were inoculated subcutaneously with 1 ml. of the same *V. fetus* suspension as employed in experiment 3. A total of five injections was administered on alternate days. Three pregnant and 1 nonpregnant animal, which served as controls,

were given 1 ml. of sterile saline on alternate days until five subcutaneous injections had been made. Serum agglutination tests were conducted in the same manner as in experiment 3.

RESULTS

Experiment 1.—Group A.— Four of 7 gravid animals aborted at one to twelve days following the last intraperitoneal inoculation. *Vibrio fetus* was isolated in pure culture from the uterus and stomach contents of 1 of the fetuses. On the ninth day, the fifth of the pregnant animals died and *V. fetus* was recovered in contaminated culture from the uterus. The sixth and seventh pregnant animals were destroyed at twenty-one and twenty-five days, respectively. Pure cultures of *V. fetus* were isolated from the former and were seen in Gram-stained slides of the uterine contents of the latter but were not demonstrable by bacteriological culture. Three nonpregnant animals were destroyed at either thirty or thirty-one days. *Vibrio fetus* was not observed in these animals.

Group B.—One of 7 pregnant animals which received *Br. abortus*, strain 19, subcutaneously aborted and Brucella organisms were isolated from the uterus and fetus. Strain 19 also was isolated at parturition from the uterus of 2 other animals at fifteen and twenty-four days. Brucella organisms were not recovered from the uteri of 3 nongravid animals but were present in the spleens of 3 and in the axillary lymph nodes of 9 of the 10 guinea pigs.

Group C.—Five of the 6 pregnant animals aborted between one and five days after the last *V. fetus* inoculation. Three uteri yielded pure cultures of *V. fetus*, while Vibrio and Brucella organisms were isolated from two. The sixth gravid animal was killed at fourteen days and *V. fetus* was isolated in pure culture. *Vibrio fetus* was observed in, but not cultured from, the uterine contents of a nongravid animal at eighteen days. Another nongravid animal yielded Vibrio and Brucella organisms upon uterine culture at twenty-two days, while from the last 2 nonpregnant animals pure cultures of *V. fetus* were isolated at twenty-two and twenty-three days. Brucella organisms were recovered from the spleens of 4 guinea pigs, 2 of which were not gravid. Brucella organisms were isolated from the axillary lymph nodes of all animals in this group.

All of the controls, group D, were bacteriologically negative on culture for Vibrio and Brucella organisms at the termination of the experiment. The results of this investigation are summarized in table 1.

TABLE 1—Results of Experiment 1

Group	No. of Animals	Status	Termination*	Uterine-fetal bacteriological examinations
A	7	Pregnant	4 aborted at 1-12 days	*Vibrio fetus* in pure culture from all 4.
			1 died at 9 days	*V. fetus* in contaminated culture.
			1 destroyed at 21 days	*V. fetus* in pure culture.
			1 destroyed at 25 days	*V. fetus* seen.**
A	3	Nonpregnant	Destroyed at 30-31 days	All negative.
B	7	Pregnant	6 destroyed at 15-34 days	Strain 19 isolated from 2.
			1 aborted at 22 days	Strain 19 isolated.
B	3	Nonpregnant	Destroyed at 31-32 days	All negative.
C	6	Pregnant	5 aborted at 1-5 days	*V. fetus* in pure culture from 3.
			1 destroyed at 14 days	*V. fetus* and strain 19 from 2.
				V. fetus in pure culture.
C	4	Nonpregnant	Destroyed at 18-23 days	*V. fetus* and strain 19 from 1.
				V. fetus seen in 1.**
				2 animals were negative.

Group A—*V. fetus* infection alone.
Group B—*Brucella abortus*, U.S.D.A. strain 19, infection alone.
Group C—*V. fetus* infection superimposed upon strain 19 infection.
Group D—controls which were comprised of 4 pregnant and 1 nonpregnant animal and which were inoculated with sterile saline intraperitoneally. All were bacteriologically negative at the termination of the experiment.
* Time after last bacterial inoculation. ** Microscopic examination of wet mount slides and Gram-stained slides of uterine and fetal materials.

Experiment 2.—One of 6 pregnant guinea pigs aborted at eight days after the last intravaginal inoculation of *V. fetus*. Lochia was noted four to five days prior to abortion. The organism was not cultured from the uterus or fetal stomach contents

but was observed in Gram-stained smears of these materials. The gross and microscopic pathological alterations observed in the uterus of this animal appeared to be identical with the changes seen in guinea pigs from which Vibrio organisms were isolated. The remaining 5 pregnant, the 4 nongravid, and the 5 control animals were negative for *V. fetus* upon bacteriological examination when destroyed at five to eighteen days after the last inoculation. The results of this experiment appear in table 2.

Experiment 3.—Two of 9 gravid animals aborted at seven and eleven days following the last oral inoculation. *Vibrio fetus* was not isolated from either animal, although the uterine Gram-stained smears of both guinea pigs contained organisms which apparently were *Vibrio fetus*. The uteri of both animals which aborted manifested the gross and microscopic pathological changes of vibriosis. The 7 other pregnant, the 1 nonpregnant, and the 4 control animals showed neither bacteriological nor pathological evidence of the presence of *V. fetus* infection.

The blood serum of all animals inoculated with *V. fetus* contained agglutinins for *V. fetus*, both strains 543 and P1, at titers of 1 : 40 to 1 : 160. The serums of 2 animals with *Vibrio fetus* agglutinin titers of partial at 1 : 40 and partial at 1 : 160 also gave a partial reaction at 1 : 10 and complete reaction at 1 : 10, respectively, with *Br. abortus* antigen.

The 4 pregnant control guinea pigs were negative on bacteriological examination. One animal's serum agglutinated the two *Vibrio fetus* antigens at the 1 : 10 level.

The results of this experiment are summarized in table 2.

Experiment 4.—Two of 7 pregnant animals aborted at four and seven days, respectively, following the last subcutaneous inoculation of *V. fetus*. The organism was isolated in pure culture from both. *Vibrio fetus* was isolated in pure culture from the gall bladder of an animal which gave birth to normal young at five days. Vibrio organisms were seen in the bile of another guinea pig at nine days but could not be isolated from this material. *Vibrio fetus* serum agglutinin titers of these animals ranged from 1 : 80 to 1 : 640. Two animals had Brucella titers of 1 : 10.

The control guinea pigs gave bacteriologically negative results. One animal, however, had a demonstrable *V. fetus* titer of 1 : 10 and an incomplete Brucella agglutinin titer at the 1 : 10 dilution. The results of this experiment are presented in table 2.

GROSS PATHOLOGICAL ALTERATIONS

A serosanguinous fluid was observed in the abdominal and thoracic cavities of the animals which aborted. Deposits of fibrin were present on the surface of the organs. The placentas were edematous and areas of necrosis, which were covered with a yellow green, caseous exudate were evident (fig. 1). In two instances, multiple cysts which varied from the size of a pinhead to that of a small pea were present at the neck of the fetal placental attachment. The uteri contained a turbid yellow- or red-colored fluid. Endometrial hemorrhage, edema, necrosis, and various degrees of in-

TABLE 2—Results of Oral, Subcutaneous, and Intravaginal Exposure of Guinea Pigs to Vibrio Fetus

Inoculated	No. of Animals	Status	Termination*	Uterine-fetal bacteriological examinations
Orally (Experiment 3)	9	Pregnant	2 abortions at 7, 11 days 7 parturitions at 3-11 days	*Vibrio fetus* seen in 1; other negative All negative
	1	Nonpregnant	Destroyed at 21 days	Negative
Subcutaneously (Experiment 4)	7	Pregnant	2 abortions at 4, 7 days 5 parturitions at 3-14 days	*V. fetus* in pure culture All negative **
	3	Nonpregnant	Destroyed at 32-33 days	All negative
Intravaginally (Experiment 2)	6	Pregnant	1 aborted at 8 days 5 destroyed at 5-18 days	*V. fetus* seen † All negative
	4	Nonpregnant	Destroyed at 20-22 days	All negative

* Time after last *V. fetus* inoculation.

** *Vibrio fetus* isolated in pure culture from the gall bladder of 1 animal and seen in the bile of another.

† Lochia present. Gross and histopathological changes indicative of *V. fetus* infection.

Control animals for each of the above groups were bacteriologically negative at the termination of the experiment.

flammatory reaction were consistent findings (fig. 2). The two gall bladders from which *V. fetus* was either isolated or seen were approximately three times their normal size and filled with bile. The surface capillaries were injected and there was evidence of acute inflammation of the mucosa.

Some of the fetal tissues, particularly the skin, were edematous (fig. 3). The abdominal and thoracic cavities contained quantities of a bloody, serous fluid. The surfaces of the splanchnic organs were covered with fibrin deposits. Hemorrhages were observed under the capsule of the liver and on the epicardium. The stomachs contained a colorless, yellow, or brown turbid fluid.

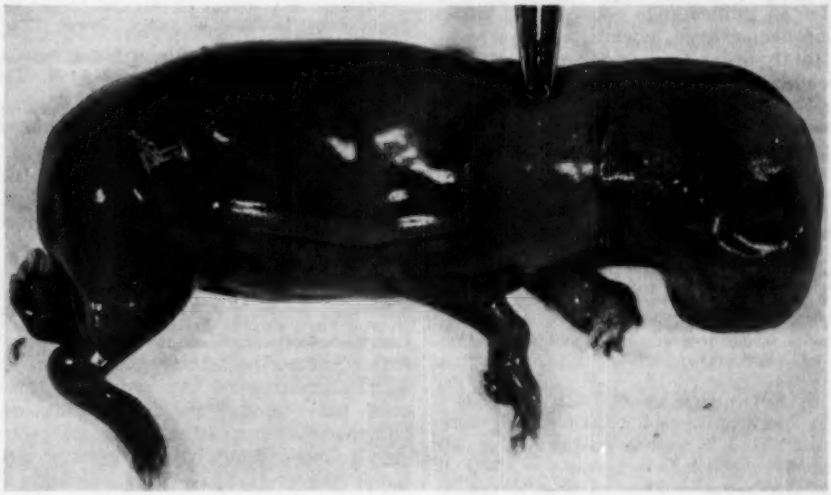

Fig. 3—Extensive edema of the skin of a fetus aborted four days after the dam was inoculated intraperitoneally with Vibrio fetus.

DISCUSSION

There was not a significant difference between the *V. fetus* infection alone as contrasted to the *V. fetus* superimposed upon the *Br. abortus*, strain 19, infection. Apparently the presence of strain 19 did not block, protect against, or markedly alter the course of the vibrionic infection and abortion. It is also of considerable interest that both Brucella and Vibrio organisms were present in the uteri of 3 animals. Apparently there is little demonstrable antagonism between the two organisms.

The intraperitoneal route of inoculation of *V. fetus* proved to be more effective in producing infection and subsequent abortion in guinea pigs than were the subcutaneous, oral, and intravaginal routes. The pathogenesis of *V. fetus* may be studied using the guinea pig as an experimental animal. Such observations can not be applied *per se*, however, to ovine and bovine vibriosis.

Exposure by the oral and intravaginal routes produced abortion in guinea pigs. The possibility is enhanced that ingestion and coitus may serve as the natural mode by which the organism is transmitted in sheep and cattle. The development of *V. fetus* serum agglutinins in guinea pigs following oral inoculation indicates the organism's parenteral penetration and its contact with the reticuloendothelial system and further indicates that ingestion might thus produce infection in cattle and sheep.

The agent is capable of residence in the nongravid guinea pig uterus, and one might assume such a situation could occur in cattle or sheep. Such evidence is indicated by the investigations of Frank.[3]

SUMMARY

The pregnant guinea pig may be infected experimentally by the intraperitoneal, subcutaneous, intravaginal, and

oral routes with *Vibrio fetus*. This organism was isolated in pure culture from the uteri and fetuses of animals which aborted. Vibrio organisms also were isolated from the nongravid uteri and gall bladders of some of the experimental animals. *Vibrio fetus* serum agglutinins were demonstrable in all animals which received oral inoculations of the organisms.

The gross pathological changes observed in infected guinea pigs consisted of endometrial hemorrhage, edema, and necrosis. The fetal organs were edematous and hemorrhagic.

References

[1] Barner, R. D., and Oberst, F. H.: Vibrionic Abortion in Cattle. Vet. Med., *45*, (1950): 389-391.

[2] Cotton, W. E.: Abortion Disease of Cattle. J.A.V.M.A., *55*, (1919): 504-528.

[3] Frank, A. H.: U.S.D.A., Animal Disease Station, Beltsville, Md. Personal Communication, 1952.

[4] Kuzdas, C. D., and Morse, E. V.: Selective Medium for the Isolation of Brucella from Contaminated Material. J. Bact. (in press).

[5] Lawson, J. R., and MacKinnan, D. J.: *Vibrio Fetus* Infection in Cattle. 70th Ann. Congress. Brit. Vet. A., (Sept. 7-13, 1952): 1-10.

[6] Lerche, P.: Beitrag zum Spirillen Abortus beim Rinde. Deutsche tierärtzl. Wchnschr., *33*, (1937): 118-119.

[7] McFadyean, F., and Stockman, S.: Report of the Departmental Committee Appointed by the Board of Agriculture and Fisheries to Investigate Into Epizoötic Abortion. London, (1913): 26-30.

[8] Plastridge, W. N., and Williams, L. F.: Observations on *Vibrio Fetus* Infections in Cattle. J.A.V.M.A., *102*, (1943): 89-95.

[9] Plastridge, W. N., Williams, L. F., Easterbrooks, H. L., Walker, E. C., and Beccia, R. N.: Vibriosis in Cattle. Bull. 281, Storrs (Conn.) Agric. Exptl. Sta., (1951): 1-51.

[10] Roberts, S. J., Gilman, H. L., and Larsen, P. H.: *Vibrio Fetus* Infection in Cattle. Cornell Vet., *40*, (1950): 111-124.

[11] Schroeder, E. C.: A Review of Several Publications on Infectious Abortion Disease. J.A.V.M.A., *57*, (1920): 270-281.

[12] Simon, J.: Vibrio Studies—Histopathological Alteration of the Bovine Uterus. Doctor of Philosophy thesis, Univ. Wisconsin, 1951.

[13] Smith, T.: The Etiological Relation of Spirilla *(V. Fetus)* to Bovine Abortion. J. Exptl. Med., *30*, (1919): 313-324.

[14] Smith, T.: Spirilla Associated with Disease of Fetal Membranes in Cattle. J. Exptl. Med., *28*, (1918): 701-719.

[15] Smith, T.: Further Studies on the Etiological Significance of *Vibrio Fetus*. J. Exptl. Med., *37*, (1923): 341-356.

[16] Wilson, W. R., and McDiarmid, A.: The Occurrence of *Vibrio Fetus* in Aborted Material Received from Cows Inoculated with Strain 19 *Brucella Abortus* Vaccine. Vet. Rec., *62*, (1950): 589-591.

Studies on the Hemoglobin Content of Sheep Blood in the Sierra of Peru

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IN THE course of the last five years in our work with sheep in the Sierra or Andes Mountain region of Peru, we have had occasion to notice that in many cases the hemoglobin readings were much higher than is usually considered normal. In many of these animals, one could readily detect the clinical symptoms of anemia. It, therefore, became apparent that a carefully controlled study of hemoglobin levels in sheep maintained at this altitude would be of benefit so that we might establish a figure which could be considered normal for this area, and for other areas of similar nature.

The work cited was done in the veterinary laboratory of the Cerro de Pasco Corporation. The animals used were selected from the 200,000 maintained by the corporation on its Ganadera Department. These sheep were of all ages; both sexes were represented, some animals were provided shelter but the greater number were living under range conditions. It should be noted that the altitude of this region is from 11,500 feet to over 15,000 feet. The rainfall is definitely divided into two periods of almost equal duration, that is, almost six months with an abundance of rain, and six months with little, or no rain.

The studies enumerated in the following paragraphs were initiated on a limited scale several years past with the use of the Spencer hemoglobinometer. Since this is an apparatus which depends on comparison of colors, it is open to a certain amount of error due to differences in visual acuity. We have not included these readings in our report. All readings reported were made with the Fisher electrophotometer. This machine was carefully calibrated against other machines of the same type. The machine was also calibrated by the method for the determination of iron in blood described by Andes and Northrup.[1] Once having determined the iron content of the sample, the hemoglobin content of the same sample may be determined, making use of the fact that hemoglobin contains 0.335 per cent iron. In this manner, a calibration factor for the individual machine was prepared for both iron and hemoglobin and a double check on the accuracy of the readings was available.

Hemoglobin readings were made by the acid hematin method. Twenty-five cc. of 0.1 N hydrochloric acid was placed in the absorption cell. By means of the Sahli pipette, 0.02 ml. of well-mixed oxalated blood was discharged into the cell, being thoroughly mixed by rinsing the pipette with the acid solution. Four minutes were allowed as the minimum for hemolysis of the sample. Readings were then made using the calibration figure and a chart prepared from this figure for the machine.

Blood was drawn once weekly from the same animals and was removed from the jugular or radial vein. The contents of the syringe were expelled into a sterile bottle in which a solution of ammonium and potassium oxalate had been evaporated. Readings were made on the same day. Factors which might influence the results were recorded and in no case were readings from sick animals utilized. Results are expressed in grams per 100 cc.

Dukes, in his "Physiology of Domestic Animals,"[2] cites Baker's normal for sheep as 11.18 Gm. per 100 cc. The revision of A. H. Craig given in Coffin's "Manual of Veterinary Clinical Pathology"[4] gives a range for sheep of 9.00 to 14.50 Gm. Reda and Hathout[5] give 8.3 Gm. for sheep infested with stomach parasites and 13.8 Gm. for sheep suffering from intestinal parasites. They gave the normal for lambs as 10.57 Gm., for young sheep as 10.80 Gm., and for adult sheep as 8.10 Gm. During the first month of pregnancy, they recorded average readings of 8.55 Gm., the second month, 9.75 Gm. At the fifth month, or at the time of parturition, they averaged 7.80 Gm. per 100 cc.

One-year-old ewes studied for twenty-eight weeks had an average hemoglobin level of 15.46. The readings ranged from a high of 16.30 to a low of 12.34 Gm. The average weekly change was plus 0.25 Gm., minus 1.05 Gm. Twenty animals were used.

Twenty 2-year-old ewes gave an average of 15.01 Gm. over the same period. The range was high at 16.10 and low at 13.74 Gm.

Twenty 4-year-old ewes gave an average of 15.15 Gm. with a high of 16.19 and a low of 13.11.

Twenty 3-year-old ewes gave an average of 15.23 Gm. with a high of 16.10 and a low of 13.15.

Twenty broken mouthed (5 years or over) ewes gave an average of 15.33 Gm. with a high of 16.37 and a low of 13.11 Gm. These were ewes of the fifth lambing.

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Fifteen yearling ewes gave an average of 15.85 Gm. on weekly blood samples taken over a period of ten months.

A similar number of yearling wethers gave an average of 15.62 Gm. over a like period.

Wethers of two years of age gave an average of 16.52 over a ten-month period.

A group of 34 lambs gave an average of 15.16 Gm. at the time of birth, with an average of 15.23 for the second week of life. The rate of gain or loss in this week was calculated per animal and it was found to be minus 0.52 Gm. for the 6 lambs that showed a reduction of Hb. For those that showed an increased Hb. level the rate was plus 0.48 for 7 lambs. It should be noted that all lambs were born and raised under average range conditions.

A group of 23 lambs whose mothers are maintained on cultivated, irrigated pasture and who were stabled for the night and provided with hay and a quarter pound of grain per day were also included in the study. These lambs gave an average of 15.82 Gm. at birth and for the first week of life an average of 16. A slight difference in favor of the lambs whose mothers received supplementary diet may be noted. The average increase of the fed group lambs over the range born lambs at time of birth was 0.59 Gm.

A characteristic graph may be prepared from the Hb. levels of all normal lambs, irrespective of their mother's food level. The level at birth always increases from 0.5 to 1.0 Gm. for a period of from six to eight weeks after birth, in many cases attaining a level of 16 to 17 Gm. At the end of the eighth to tenth week of life the level begins to fall toward what appears as the level for adults.

In a group of 18 ewes of various ages, an effort was made to determine what changes would be noted in the hemoglobin level when these ewes were changed from cultivated pasture to range pasture. The average hemoglobin level of these ewes on cultivated pastures for ten weeks was 15.17 Gm. At the day of the change to range the average was 15.36 Gm. One week after the ewes had been moved to range pasture the level was 15.45 Gm. Eleven ewes showed an increase, the average being 0.55 Gm. Four ewes showed an average decrease of 0.66 Gm. Over the following month, 8 ewes showed an average increase of 0.64 Gm. Ten demonstrated a decreased level, with an average loss of four weekly readings of 0.55 Gm. No significant change can be demonstrated.

The same group of ewes that were used to establish a normal for various ages were then studied as they lambed. All the readings taken during the last three months of pregnancy were taken as the average before lambing and given as such. The readings of all animals were averaged the week before lambing, the week of lambing, and the week after lambing. Average gains and losses for each group were calculated (*see* table 1).

Several ewes which had been included in the original work, to obtain a normal, became ill, 3 from respiratory infections and 1 from vaginal prolapse. These ewes were removed from the flock and hospitalized. The readings taken on these animals were excluded entirely from consideration but are noted here to illustrate the changes observed. The readings of the eight to ten weeks before illness intervened averaged 15.55 Gm. The week before illness was 14.92, the week of acute illness was 7.19 Gm., and the week when recovery began was 12.57 Gm. The average of the weeks after illness was 13.55 Gm. on an average of five readings per animal. This illustrated the rapid change in the Hb. level in the course of disease and recovery. All of these ewes lambed normally during recovery and the state of health of ewes and lambs is good.

SUMMARY

We have presented the results of some work done on sheep blood with particular reference to hemoglobin levels. This work was done on animals of both sexes, various age levels, and under different condition of nutrition, all animals living at an altitude of from twelve to fifteen thousand feet. Changes taking place during pregnancy, preparturition, and postparturition

TABLE 1—Average Gains and Losses in Hemoglobin Content of Pre- and Postparturient Ewes, in Grams

Age of ewes	Ave. for 10 wks. preparturient	One wk. before lambing	Change of week before lambing	Week of lambing	One wk. after lambing	Change of week after lambing
Yearling	14.81	14.38	+0.48 −0.91	14.19	14.31	+0.53 −0.0
Two year old	15.05	14.74	+0.60 −0.45	14.89	14.73	+0.36 −0.61
Three year old	15.12	14.58	+0.38 −0.18	14.71	15.28	+0.68 −0.0
Four year old	14.68	13.58	+0.93 −0.30	14.09	15.46	+1.18 −0.0
Aged (fifth lamb)	14.79	14.09	+0.71 −0.30	14.01	15.46	+1.18 −0.0

were recorded as well as changes during illness and recovery from disease.

On several occasions we have imported sheep from areas at or a little above sea level. These animals always arrive with a Hb level of from 8.0 to 10.0 Gm. One may note a steady climb and, over a period of three to four months, we find that they have reached the levels we have given above. This is quite similar to the results of studies on man.

References

[1] Andes, J. E., and Northup, D. W.: Photoelectric Colorimetry: Determination of Blood Iron and Hemoglobin. J. Lab. and Clin. Med., *24*, (1938): 197-206.

[2] Wong, S. Y.: Colorimetric Determination of Iron and Hemoglobin in Blood. J. Biol. Chem., 77, (1928): 409.

[3] Dukes, Henry H.: Physiology of Domestic Animals. Comstock Publishing Co. 1943.

[4] Coffin, David L.: Manual of Veterinary Clinical Pathology. Comstock Publishing Co. 1945.

[5] Reda, H., Hathout, S. F.: Hematological Examination of Blood of Sheep under Physiologic and Pathologic Conditions. J.A.V.M.A., *118*, (1951): 253.

The Immunization of Young Lambs Against Enterotoxemia

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ALTHOUGH type D *Clostridium perfringens* bacterin is used extensively as a preventive for enterotoxemia, there is little evidence as to how early in the life of an animal it may be used. Gill,[1] in 1936, reported that immunization during the first week of life lowered the death rate from enterotoxemia from 2.2 to 1.25 per cent in a large group of lambs but did not consider this to be a significant difference. Since present reports from the field vary, some practicing veterinarians reporting satisfactory results from active immunization during the first few weeks of life and others using only passive immunization during this period, it was decided to investigate the immunity response of young lambs treated with commercial type D *Cl. perfringens* bacterin. Because it is not possible to reproduce this disease with certainty, direct protection tests could not be done and, instead, immunity response was measured by the titration of antitoxin to *epsilon* toxin in the circulating blood.

Also, a few experiments were performed in an attempt to verify, with *epsilon* toxin, the finding of Nicholson [2] that the injection of sublethal doses of type D *Cl. perfringens* culture filtrates conferred almost immediate protection against the subsequent injection of several certainly lethal doses of this same material. Such a phenomenon, if reproducible, would offer a method of conferring immunity which would be both immediate and long lasting.

MATERIALS AND METHODS

The standard toxin for this experiment was prepared from the filtrate of a broth culture of type D *Cl. perfringens*, the toxic dose for mice of which was less than 0.0001 ml., and more than 0.00005 ml., after trypsin activation. The filtrate was saturated with ammonium sulfate, let stand at room temperature for one hour, and the scum removed. The fluid portion was centrifuged, the supernatant fluid discarded, and the precipitate was added to the scum. This mixture was dried in a vacuum desiccator over calcium chloride, powdered by grinding in a mortar, and stored at −36 C. The lethal dose for white mice by intravenous injection was 0.001 mg.; the 1+ dose was 0.250 mg.; the l_f value was 0.225 mg. The 1+ and l_f values were determined by titration against a sample of standard serum containing 500 Wellcome units per milliliter, received from Mrs. I. Batty of the Wellcome Research Laboratories. The procedure followed was that of Batty and Glenny.[3]

The lamb serums were tested for antitoxin activity by mixing 1.3 ml. of serum with 0.2 ml. of diluted toxin. The latter was prepared by dissolving 5.0 mg. of dry toxin in 120 ml. of a solution of 0.85 per cent NaCl and 0.2 per cent bactopeptone. Such mixtures were allowed to stand for thirty minutes at room temperature, then 0.5 ml. of each was injected intravenously into each of 2 mice, which were held for four days. If the mice survived, lesser amounts of serum were used until the amount of serum was determined which was just insufficient to protect the mice. Toxin solutions were always used within one hour of the time that they were made up. Results of these titrations are given in terms of Wellcome units; this standard was set up in 1933. One Wellcome unit of antitoxin is equal to approximately 8.3 of the antitoxin units used by Baldwin *et al.*[4] in their work on the immunization of feedlot lambs. The highest titer found at any time subsequent to immunization was used as the index of the immunity attained.

Four groups of 10 lambs each were injected subcutaneously with 3.0 ml. of commercial type D *Cl. perfringens* bacterin. This material was an alum-precipitated formolized whole culture. It had been treated with trypsin in the course of preparation. The lambs were bled just previous to immunization and at weekly intervals thereafter for three weeks. Group 1 consisted of animals immunized at about 10 days of age; group 2 of animals immunized at about 20 days of age; group 3 about 30 days of age; and group 4 about 60 days of age.

EXPERIMENTAL RESULTS

Before the immunization of lambs was attempted, an effort was made to repeat the results of Nicholson, with regard to the establishment of resistance by the injection of sublethal doses of toxin. White mice were injected intravenously with 0.8, 0.4, 0.2, and 0.1 μg. of toxin. One day later, these animals were injected with 3.3 μg. of toxin; all died. The injection of commercial type D *Cl. perfringens* bacterin, in doses ranging from 0.3 to 0.001 ml., did not

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confer any appreciable resistance to subsequent doses of toxin. It is probable that the immediate resistance noted by Nicholson was due not to *epsilon* toxin in his culture filtrates but to *theta* toxin, which is also produced by type D strains. Since the multiplicity of *Cl. perfringens* toxins was not recognized at the time that he did his work, it was not possible for him to determine how much of the toxicity of his culture filtrates was due to *alpha* toxin, how much to *epsilon* toxin, and how much to *theta* toxin. When tryptic conversion of prototoxin to *epsilon* toxin is used, as it was in the present experiment, the other toxic components are very largely inactivated.

From the results of the experiment on the immunization of lambs given in table 1, it is notable that 4 of 40 lambs were found to possess appreciable concentrations of antitoxin in the circulating blood before immunization. Three of these animals, Z-132, Z-139, and Z-96, probably were passively immune, for they did not show any greatly increased antitoxin production after stimulation with *Cl. perfringens* bacterin. Animal Z-45 may well have been one which had suffered from a subclinical infection with type D *Cl. perfringens*, for it immediately responded to a single immunizing dose with an antitoxin titer as high as that of any animal investigated. The ewes which gave birth to lambs Z-96 and Z-139 had antitoxin titers of 0.21 and 0.052 units, respectively, two months after lambing. The ewes which gave birth to Z-132 and Z-45 had no detectable antitoxin in their circulating blood.

Only 2 of 9 animals immunized at 10 days of age showed appreciable levels of antitoxin; the mean of the highest values attained being 0.023 unit per milliliter. Of the animals immunized at 20 days of age, 8 of 10 showed appreciable antitoxin, with a mean value of 0.40 units per milliliter, while of those immunized at 30 days of age, 6 of 10 showed appreciable antitoxin, with a mean value of 0.72 units. The proportions of animals showing immunity response in these latter two groups are reversed from what might be expected, for the younger group had a greater number of immune individuals. No explanation for this discrepancy can be offered other than the probable variability of such young animals. All of the animals in the group immunized at 60 days of age showed an immunity response, with a mean value of 1.26 units per milliliter. This group served to demonstrate the antigenic efficacy of the immunizing agent used. It is not certain that serums were taken at the time the immunity response was greatest. The fact that 20 of the 27 immune animals showed a higher antitoxin titer at 3 weeks than they had previously indicates that the peak of immunity response may not have been reached by this time.

It is apparent from these results that an appreciable proportion of animals as young as 20 days of age can be immunized

TABLE 1—Antitoxin Titers of Lambs Immunized at 10, 20, 30, and 60 Days of Age Titers Expressed in Wellcome Units

Lamb (No.)	Preimmunization bleeding	Postimmunization bleedings		
		1 week	2 weeks	3 weeks
Group 1—10 Days of Age				
Z-126	NDA	NDA	NDA	NDA
Z-128	NDA	NDA	NDA	NDA
Z-132	0.026	(died in course of experiment)		
Z-138	NDA	NDA	NDA	NDA
Z-139	0.21	0.026	0.052	0.052
Z-143	NDA	NDA	NDA	NDA
Z-144	NDA	NDA	NDA	0.10
Z-145	NDA	NDA	NDA	NDA
Z-151	NDA	NDA	NDA	NDA
Z-154	NDA	NDA	NDA	NDA
Group 2—20 Days of Age				
Z-96	0.026	NDA	0.026	0.026
Z-97	NDA	0.83	3.3	3.300
Z-104	NDA	0.026	0.026	0.052
Z-107	NDA	NDA	0.10	0.210
Z-115	NDA	NDA	NDA	NDA
Z-117	NDA	0.026	NDA	0.210
Z-119	NDA	NDA	0.026	NDA
Z-121	NDA	NDA	NDA	0.100
Z-123	NDA	NDA	0.052	0.100
Z-125	NDA	NDA	NDA	0.026
Group 3—30 Days of Age				
Z-70	NDA	0.21	0.42	0.830
Z-73	NDA	NDA	0.026	0.100
Z-75	NDA	NDA	NDA	0.100
Z-77	NDA	NDA	NDA	0.100
Z-78	NDA	NDA	NDA	0.100
Z-80	NDA	NDA	NDA	NDA
Z-82	NDA	NDA	NDA	NDA
Z-83	NDA	NDA	NDA	NDA
Z-85	NDA	6.0	1.7	1.700
Z-86	NDA	NDA	NDA	NDA
Group 4—60 Days of Age				
Z-31	NDA	NDA	0.10	0.026
Z-41	NDA	NDA	NDA	0.100
Z-43	NDA	1.7	0.83	0.830
Z-44	NDA	0.026	0.026	0.100
Z-45	0.15	6.0	6.0	6.000
Z-47	NDA	NDA	NDA	3.300
Z-51	NDA	NDA	NDA	0.026
Z-58	NDA	NDA	NDA	0.026
Z-62	NDA	NDA	NDA	0.026
Z-65	NDA	NDA	NDA	0.052

NDA—No detectable antitoxin.

to some degree against the *epsilon* toxin of type D *Cl. perfringens*. It is also apparent that immunization at this age, with the antigen that we used, can not be relied upon to provide immunity in all animals of this age. All the lambs which were about 60 days of age, however, responded to the single immunizing dose with the production of a measurable amount of antitoxin. Since no direct evidence is available on the level of antitoxin needed to protect against enterotoxemia, it is not possible to state with certainty that immunity such as encountered in this experiment would be sufficient to protect against enterotoxemia. However, to judge from the protection given young lambs in the field by the injection of immune serum, 0.3 units of antitoxin per milliliter suffices to give adequate protection, and it is probable that appreciably lower concentrations are sufficient to give protection against enterotoxemia. It is possible that the greatest benefit of immunization against *epsilon* toxin may not be merely the attainment of an appreciable antitoxin titer shortly after immunization but, rather, the attainment by the animal of a state of immunity sufficient to ensure the production of protective amounts of antitoxin should an infection with *Cl. perfringens* take place subsequently.

SUMMARY

No immediate protection was afforded to white mice against the *epsilon* toxin of type D *Clostridium perfringens* by the injection of sublethal doses of toxin or by injection of commercial bacterin.

Antitoxin was demonstrated in the circulating blood of 2 of 9 lambs immunized at 10 days of age with commercial bacterin; in 8 of 10 immunized at 20 days of age; in 6 of 10 immunized at 30 days of age; and all of 10 immunized at 60 days of age. Considerable variation was found in immunity response from one animal to another.

These results indicate that vaccination of lambs at 10 days of age with a type D *Cl. perfringens* bacterin is of little value; that perhaps 60 to 80 per cent of lambs 20 to 30 days of age will show immunity response; and that all lambs 60 days of age may be expected to respond in some degree. In judging the value of vaccination of young lambs against enterotoxemia, it should also be noted that in 50 per cent of the lambs showing immunity response, the antitoxin was not demonstrable in the blood until three weeks after vaccination.

References

[1] Gill, D. A.: Infectious Enterotoxemia and the *Clostridium Welchii* Group, with Special Reference to So-Called Pulpy Kidney in Lambs. New Zealand J. Sci. Tech., *18*, (1936): 106-109.

[2] Nicholson, J. A.: Some Observations on the Physiological Actions of *Clostridium Welchii* Toxins. Fourth report of the Director, University of Cambridge Inst. of Anim. Path., (1934-35): 212-226.

[3] Batty, I., and Glenny, A. T.: Titration of *Clostridium Welchii* Epsilon Toxins and Antitoxin. Brit. J. Exptl. Path., *27*, (1947): 110-126.

[4] Baldwin, E. M., Frederick, L. D., and Ray, J. D.: The Control of Ovine Enterotoxemia by the Use of *Clostridium Perfringens* Type D Bacterin. Am. J. Vet. Res., *9*, (1948): 296-303.

Efficacy of Estradiol Cyclopentylpropionate (ECP) in Anestrous Ewes

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NUMEROUS attempts have been made to induce estrus and ovulation in anestrous ewes with the thought of eventually increasing lamb production and employing controlled breeding. Various hormones and hormonal combinations have been employed as indicated by the review paper of Phillips *et al.*[22] It seems advisable to review previous results obtained with estrogens by other workers, since this report deals with a recently developed estrogen in an attempt to induce estrus and ovulation in anestrous ewes.

REVIEW OF LITERATURE

Cole and Miller[8, 9] employed estrin in anestrous ewes and concluded that this estrogenic hormone inhibited ovulation.

McKenzie and Terrill[20] produced estrus in 44 per cent of 52 ewes injected once with 800 rat units of estradiol benzoate (progynon-b). Estrus occurred on the average within fifty hours following the injection into the anestrous ewe.

Bell *et al.*[4] induced estrus but no ovulation in 5 out of 8 ewes using estradiol benzoate.

Anderson[3] injected estradiol benzoate into anestrous lactating Merino ewes and produced estrus in 74.3 per cent of them, but subsequent ovulation did not always occur.

The most satisfactory results have been reported by August[2] who used estradiol benzoate in anestrous ewes and caused 72.5 per cent of 4,281 treated ewes to mate and 43.1 per cent to lamb.

Frank and Appleby[15] induced estrus in 11 of 12 anestrous ewes by injecting stilbestrol. When combined with pregnant mares serum (pms), only 7 of 15 treated animals ovulated. When pms was employed alone, all 13 ewes treated ovulated. These workers concluded that stilbestrol alone, or in combination with pms, did not provide satisfactory hormonal stimulation for successful breeding in anestrous ewes.

Hammond, Hammond, and Parkes[16] used stilbestrol and estradiol in anestrous ewes with poor results. Ten ewes received stilbestrol and 6 exhibited estrus and 6 ovulated, but estrus and ovulation occurred simultaneously in only 3.

Quin and Van Der Wath[25] treated 43 anestrous ewes with stilbestrol and were able to induce estrus and ovulation in some cases.

Hammond, Hammond, and Parkes,[16] and Quin and Van Der Wath[25] pointed out that it is probable that some, and perhaps all, of the ovulations following the use of naturally occurring estrogens did not result from the injections but resulted from "silent estrus" during the nonbreeding season. These workers thought that perhaps stilbestrol and possibly other estrogens could induce ovulation by stimulating release of gonadatropins from the pituitary gland.

The interrelationship as it is presently understood between estrogens and the pituitary gland hormones will be discussed under the heading "Data and Discussion."

MATERIAL AND METHODS

This experiment was designed to evaluate the use of the estrogenic preparation estradiol cyclopentylpropionate (ECP *) under field conditions. Two privately owned flocks were selected. It was not possible to perform laparotomies to determine evidence of ovulation. The ECP was measured in milligrams (2 mg./cc.). All injections were administered intramuscularly in the cervical region.

Injections were conducted during the spring and summer of 1952. Seventy-seven ewes, 4 rams, and four breeds (table 1) were employed in this experiment. The Suffolk and Cheviot ewes were suckling lambs at the time of injection. The Shropshire ewes had not lambed in 1952. The Southdown ewes consisted of 3 that had lambed late in 1952 and three that had not lambed in 1952. All ewes and rams were in excellent physical condition at the time of injection.

The injection of ewes was timed so that a ram would not have more than 6 to breed during any one four-day period.

DATA AND DISCUSSION

The dosage of ECP employed varied from 2 mg. to 8 mg., but no apparent difference in the estrogenic response was noticeable. The 2-mg. dose was used in 54 of the 77 ewes injected. Estrus was induced in 90.9 per cent of the ewes following a single injection of ECP (table 1).

Rams were permitted to run with the treated ewes. All ewes were bred as they came into estrus. Many were bred twice during the estrous period.

Twelve of the 77 ewes treated exhibited estrus in twenty-four hours following the injection of ECP; 41 in forty-eight hours;

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* ECP is manufactured and marketed by The Upjohn Company, Kalamazoo, Mich.

13 in seventy-two hours; 4 in ninety-six hours, and 7 failed to exhibit estrus. The average length of time from the injection of ECP until estrus was exhibited is shown in table 1, along with the breed and the dosage employed.

Only 1 of the 77 ewes treated with a single injection of ECP gave birth to a lamb. The lamb was stillborn and arrived on the 147th day of gestation. One ewe developed constant estrus following the estrogen treatment.

TABLE 1—Average Length of Time from Injection of ECP until Estrus

Breed	Age (yr.)	No. of ewes	Dose (mg.)	Ave. hours injection to estrus	No. ewes showing no estrus	No. ewes lambing
Suffolk	2	5	2	43	1	0
	2	5	3	48	2	0
	2	5	4	48	3	0
	2	1	6	48	0	0
	2	1	8	48	0	0
Cheviot	2	17	2	45	0	0
	2	8	4	51	0	0
	2	3	6	56	0	0
Shropshire	2 to 3	26	2	49	1	1
Southdown	2	6	2	60	0	0
Totals		77			7	1

Estrogens, or estrogenic hormones, are so designated because of their capacity to induce estrus in the immature female, ovariectomized adult, or mature female in anestrus. The physiological effects of the estrogens simulate the action of the ovary during the follicular phase of the estrous cycle, producing growth in the vaginal and uterine walls, the mammary glands, and secondary sex characteristics.

The ovary is regarded as the principal source of estrogen within the organism; however, significant amounts may be isolated from placentas, testes, adrenal cortices, and certain plant tissues. The following are considered naturally occurring estrogenic compounds: (1) estrone or theelin, and estrone sulfate; (2) estriol or theelol, and estriol glycuronide: (3) estradiol (*alpha* and *beta* isomeric forms), or dihydrotheelin; (4) equilin, hippurin, equilenin, and *alpha* dihydroequilenin.

A number of compounds possessing estrogenic properties, that do not occur naturally within the organisms, have been prepared synthetically by organic chemists.[12, 13, 17, 18] Among the more commonly employed synthetic estrogens are diethylstilbestrol and dihydrodiethylstilbestrol or hexestrol. These two compounds have phenolic properties but, like the natural estrogens, possess estrogenic potency that is equal to or in excess of that of estradiol. Diethylstilbestrol is relatively inexpensive.

Estradiol is considered the most potent natural estrogen and apparently is the compound actually secreted by the ovary. Chemically, it is quite similar to estrone, the first estrogen isolated in crystalline form by Doisy.[11] Estradiol differs chemically in that it contains a hydroxyl group at position 17 instead of a ketone group. The *alpha*-estradiol is approximately 30 times more potent than the *beta*-estradiol.

Estriol is the principal estrogen isolated from the urine of pregnant women. It has also been isolated from the placenta. Chemically, it contains 1 molecule more of water than estrone and can be converted to estrone by dehydration *in vacuo*.[10] Generally, it is agreed that estriol is an excretory product and usually occurs in conjugation with glycuronic acid.[7] The resulting product is estriol glycuronide, an estrogen with little physiological potency.

Estradiol cyclopentylpropionate (ECP) is the oil-soluble 17 *(beta)*-cyclopentylpropionate ester of *alpha*-estradiol.

What is the effect of estrogens on the ovary? Can estrogens influence follicular growth and ovulation?

Originally, it was thought by most endocrinologists that hormones did not physiologically affect the tissues of the glands which elaborate them. When considering the ovary, this concept must be modified in accord with recent experimental observations. Estrogens injected in mice and fish stimulate mitotic proliferation of the germinal epithelium and the rate of oögenesis.[5, 6, 23] It seems reasonable to suppose that the estrogen contained in the mam-

malian follicle stimulates mitosis in the follicular granulosa cells.

Diethylstilbestrol pellets implanted subcutaneously into immature female rats two days following hypophysectomy either prevent or retard ovarian atrophy.[21, 27, 28] The response of the ovaries in the hypophysectomized rat following the administration of estrogen appears to be due to a mild growth effect on many follicles, but it is not sufficient to induce antra formation in such follicles.[24]

In adult animals, the injection of estrogens during proestrus or estrus will result in an increase in ovarian weight. The ovarian weight increase is due to the formation of corpora lutea resulting from the stimulation of the luteinizing fraction of the anterior pituitary.[20] Experimental evidence seems to show that estrogens cause the anterior pituitary gland to release an increased amount of luteinizing hormone (lh) and that lh synergizes with the follicle stimulating hormone (fsh) to carry the mature follicle through ovulation and finally corpus luteum formation. The lh from the pituitary causes ovarian hyperemia and appears to act as a maturation factor for the ovarian stroma, theca interna, and granulosa. It also stimulates the elaboration of estrogen.

Thus far, the thesis indicates that lh and estrogen stimulate each other. The physiological process involved is not a simple one. Luteinizing hormone must have the synergism of fsh and, apparently, the lactogenic hormone secreted by the pituitary gland for estrogen secretion; estrogen in turn probably causes increased lh release through certain oxidation products. Animal experiments support the concept that oxidation products of the estrogens rather than estrogens as such probably cause the increased release of pituitary lh.

An oxidation product of crystalline estrone has been prepared which is much less estrogenic than estrone but more effective than estrone in stimulating the release of lh in rats. This product is rendered highly estrogenic by hydrogenation.[29]

On the other hand, it is generally recognized that estrogens administered clinically or experimentally to an animal over a prolonged period in sufficiently large dosage will cause inhibition of pituitary fsh and inactivation of the ovaries. The underlying mechanism of this reaction is obscure, probably caused by the intact estrogen molecule.

Physiologically, estrogens appear to have a twofold effect on the anterior pituitary: (1) stimulation of lh by certain oxidation products of the molecule, and (2) fsh inhibition by the intact estrogen molecule.

There is also circumstantial evidence which indicates that the secretion of the pituitary lactogenic hormone is stimulated by oxidation products of the estrogens. Therefore, it appears that lh and the lactogenic hormones are released by estrogen stimulation of the pituitary.

Observations in animals indicate that lh plus fsh can bring about ovulation and corpus luteum formation but do not maintain luteal function, namely, the secretion of estrogen and progesterone. A third hypophyseal gonadotropin, called luteotropin (fraction of sheep pituitary extract), has been found to be involved in the regulation of the reproductive cycle of the rat.[2, 26] Pituitary luteinizing hormone stimulates the proliferation of luteal tissue but does not cause the release of progesterone by the corpus luteum.[14] In the hypophysectomized rat, the persistent corpora lutea in the ovaries do not secrete detectable amounts of progesterone. The administration of luteotropin extract does cause such corpora lutea to secrete.

The lactogenic hormone of the anterior pituitary also produces luteotrophic effects. Present evidence indicates that luteotropin and the lactogenic hormone are identical. Each of these hormones is capable of maintaining luteal function, each hormone is released by estrogen stimulation of the pituitary, and neither hormone has an effect on the ovaries without the synergism of fsh and lh.

Summary and Conclusions

Estrus may be induced in ewes during their anestrous season by the single injection of 2 mg. or more of the estrogenic preparation, estradiol cyclopentylpropionate (ECP). Of 77 ewes in anestrus treated with a single injection, 90.9 per cent came into estrus.

Under the conditions of this experiment ECP was not effective in initiating estrus and ovulation. Only 1 ewe lambed out of 77 treated. It would appear that this ovulation following the use of ECP did not

result from the injection but from a spontaneous ovulation during the nonbreeding season.

Within the limits of cited experimentation, ECP, alone in single injection of from 2 mg. to 8 mg., does not appear to induce in anestrous ewes the definite hormonal stimulation of normal estrus essential for satisfactory fecundation.

The physiological role of estrogens in reproduction is discussed.

References

[1] Anderson, J.: The Induction of Oestrus in the Ewe. Vet. J., *94*, (1938): 328-334.

[2] Astwood, E. B.: The Regulation of Corpus Luteum Function by Hypophyseal Lutеotrophin. Endocrinology, *28*, (1941): 309-320.

[3] August, H. M.: Brunsterzeugung bei Schafen mit Geschlechtshormonen. Zuchtungskunde, *16*, (1941): 41-60. (Abstracted in Vet. Bull, *12*, [1942]: 392.)

[4] Bell, T. D., Casida, L. E., Bohstedt, G., and Darlow, A. E.: Production of Heat and Ovulation in the Anestrous Ewe. J. Agric. Res., *62*, (1941): 619-625.

[5] Bullough, W. S.: The Method of Growth of the Follicle and Corpus Luteum in the Mouse Ovary. J. Endocrinol., *3*, (1942): 150-156.

[6] Bullough, W. S.: Gametogenesis and Some Endocrine Factors Affecting It in the Adult Minnow (Phoxinus Laevis L.). J. Endocrinol., *3*, (1942): 211-219.

[7] Cohen, S. L., Marrian, G. F., and Odell, A. D.: Estriolglycuronide. Biochem. J., *30*, (1936): 2250.

[8] Cole, H. H., and Miller, R. F.: The Effect of Gonad-Stimulating Hormone, of Oestrin, and of the Two Combined Upon the Ewe During Anestrum. Anat. Rec., *58*, (1934): supplement 56. (Abstract).

[9] Cole, H. H., and Miller, R. F.: The Changes in the Reproductive Organs of the Ewe with Some Data on Their Control. Am. J. Anat., *57*, (1935): 39-97.

[10] Curtis, J. M., MacCorquodale, D. W., Thayer, S. A., and Doisy, E. A.: A Study of Theelin Prepared from Human and Mare Urine and from Theelol, with Some Remarks on the Preparation of Theelin from Mare Urine. J. Biol. Chem., *107*, (1934): 191-205.

[11] Doisy, E. A., Veler, C. D., and Thayer, S. A.: Follicullin from the Urine of Pregnant Women. Am. J. Physiol., *90*, (1929): 329-330.

[12] Doisy, E. A.: The Estrogens. Endocrinology, *30*, (1942): 933-941.

[13] Doisy, E. A.: The Metabolism of Estrogens. Biol. Symp. Jaques Cattell Press, *9*, (1942): 21-40.

[14] Evans, H. M., Simpson, M. E., Lyons, W. R., and Turpeinen, K.: Anterior Pituitary Hormones which Favor the Production of Traumatic Uterine Placentomata. Endocrinology, *28* (1941): 933-945.

[15] Frank, A. H., and Appleby, A.: Induction of Estrus and Ovulation in Ewes During Their Anestrus Season. J. Anim. Sci., *2*, (1943): 251-258.

[16] Hammond, J. Jr., Hammond, J., and Parkes, A. S.: Hormonal Augmentation of Fertility in Sheep. I. Induction of Ovulation, Superovulation, and Heat in Sheep. J. Agric. Sci., *32*, (1942): 308-323.

[17] Koch, F. C.: Chemistry of Hormones. Symp. on Quant. Biol., Cold Springs Harbor, L. I., New York, *10*, (1942): 61-69.

[18] MacCorquodale, D. W.: The Chemistry of the Sex Hormones. Endocrinology, *25*, (1939): 417-422.

[19] McKenzie, F. F., and Terrill, C. E.: Estrus, Ovulation, and Related Phenomena in the Ewe. Missouri Agric. Exper. Sta. Res. Bull., (1937): 264.

[20] Meyers, R. K., and Herts, R.: Effect of Estrone on Secretion of the Gonadotropic Complex as Evidenced in Parabiotic Rats. Am. J. Physiol., *120*, (1937): 232.

[21] Penchars, R. I.: Effects of Estrogens and Androgens Alone and in Combination with Chorionic Gonadotropins on the Ovary of the Hypophysectomized Rat. Science, *91*, (1940): 554-555.

[22] Phillips, R. W., Fraps, R. M., and Frank, A. H.: Hormonal Stimulation of Estrus and Ovulation in Sheep and Goats. Am. J. Vet. Res., *6*, (1945): 165-179.

[23] Quin, J. I., and Van der Wath, J. G.: The Effects of Diethyl-Stilbestrol and Pregnant Mare Serum on the Oestrous Cycle of Merino Ewes. Onderstepoort J. Vet. Sci. and Anim. Indust., *18*, (1943): 139-147.

[24] Simpson, M. E., Evans, H. M., Fraenkel-Conrat, H. L., and Li, C. H.: Synergism of Estrogens with Pituitary Gonadotropins in Hypophysectomized Rats. Endocrinology, *28*, (1941): 37-41.

[25] Stein, K. F., and Allen, E.: Attempts to Stimulate Proliferation of the Germinal Epithelium of the Ovary. Anat. Rec., *82*, (1942): 1-9.

[26] Sydnor, K. L.: Time Relationship of Deciduomata Formation in Prolactin Treated and Normal Pseudopregnant Rats. Endocrinology, *28*, (1941): 933-945.

[27] Williams, P. C.: Ovarian Stimulation by Oestrogens. Effects in Immature Hypophysectomized Rats. Proc. Roy. Soc., London, B., *132*, (1944): 189-199.

[28] Williams, P. C.: Studies on the Biological Action of Serum Gonadotrophin. II. Ovarian Response after Hypophysectomy and Estrogen Treatment. J. Endocrinol., *4*, (1945): 131-136.

[29] Williams, R. H.: Textbook of Endocrinology, (1950): 356. W. B. Saunders Co. Philadelphia.

A Study of Encephalitis in Swine in Relation to Hog Cholera

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THE INFECTIOUS encephalitides of man, some laboratory animals, and several common farm animals, particularly the horse and cow, have been the subject of intensive research during the past half century. However, little particular attention had been paid, until recently, to the disease entities which account for encephalitic symptoms in swine.

Swine are known to be naturally susceptible to rabies, pseudorabies, infectious porcine encephalomyelitis (Teschen's disease), and to louping ill by experimental intracerebral inoculation. In the United States, hog cholera is evidently the most common cause of encephalitic symptoms in swine. It has been our experience that the presence of this disease is often inapparent, particularly in suckling pigs, since the classical macroscopic lesions of hog cholera are not obvious in pigs examined early in outbreaks of a rapidly spreading encephalitic disease. However, in the majority of such outbreaks macroscopic lesions of hog cholera are recognized in the later stages of the outbreak.

The appearance of more or less severe nervous symptoms in varying proportions of swine herds recently immunized by the serum-virus method has often been reported by veterinarians and swine raisers. The encephalitic effect of this method of immunization had not been investigated until recently. The interpretation of nervous symptoms and of inflammatory lesions of the brain of the pig has often been difficult.

REVIEW OF THE LITERATURE

The regularity of the appearance of inflammatory lesions of the brain in swine experimentally inoculated with hog cholera virus was reported by Seifreid[10] in 1931. He found 30 of 39 infected swine had inflammatory brain pathology. Helmboldt and Jungherr[6] called attention to the value of neuropathological examination as an aid in the differential diagnosis of swine diseases, particularly in the diagnosis of ill-defined hog cholera. Kernkamp and Fenstermacher[8] did not refer to neuropathological examination in describing methods of diagnosis for "subtypical" hog cholera. Doyle[4] failed to find brain lesions in an apparently infectious posterior paralysis of suckling pigs, although lesions were found in the spinal cord.

Ray[9] recovered the virus of pseudorabies from the brains of swine which were involved in a spreading encephalitic disease. Later[11] he was reported to have isolated pseudorabies virus from a commercial hog cholera virus. Shope[12] described possible methods of spread of this disease in swine.

Bacterial-induced encephalitides have been more rarely reported. Creech[3] isolated *Brucella suis* from a necrotic brain lesion of a sow which was not known to have exhibited symptoms of encephalitis. Biester and Schwarte[1] isolated *Listerella monocytogenes* from the brains of swine which had exhibited encephalitic symptoms in the field.

Encephalitis of dietary origin has been suggested in swine but has not been demonstrated beyond doubt.

EXPERIMENTAL METHODS

Descriptions of Categories of Swine Brains.—Brains of 171 swine from three different sources were examined. The first source was naturally diseased swine submitted to the diagnostic laboratory. The brains of 100 such swine were examined, 38 of which were from positive or suspected cases of hog cholera as indicated by gross lesions and/or history or symptoms. The other 62 brains were from swine in which diagnoses other than hog cholera were made, and included 8 in which no diagnosis was made. This group is subsequently referred to as "category 1."

Brains of 52 swine inoculated with hog cholera virus six days previous to being killed for the production of commercial vaccinating virus were examined. All 52 had shown some degree of gross pathology, usually slight, indicative of hog cholera. This group is subsequently referred to as "category 2."

Brains of 19 additional swine, 12 of which had been inoculated with virulent hog cholera virus and immune serum three to thirty-one days prior to being killed in pairs, 3, 8, 13, 18, 23, and 31 days after inoculation were examined. Six of the others were taken from swine kept as uninoculated controls, housed separately from those vaccinated, and killed singly at the above mentioned intervals. The seventh was from the pig used to test the virulence of the virus. This group is subsequently referred to as "category 3."

Age, Weight, and Vaccination.—Swine of category 1 ranged in age from less than 1 month to over 1 year. The majority (74 of 100) were between 1 month and 6 months of age. Of the 38 swine diagnosed as having hog cholera, 28 had not been vaccinated, or had been vaccinated with a

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killed product, or had a questionable vaccination history; 5 had been vaccinated a week or more prior to examination, 5 had been vaccinated less than a week earlier. Twenty-six of the 62 non-cholera cases were definitely known not to have been vaccinated. Eight had a questionable vaccination history or had been vaccinated with a killed product. Ten had been vaccinated between one week and one month prior to autopsy; 9 had been vaccinated less than one week prior to autopsy; 9 had been vaccinated over one month prior to autopsy by the serum-virus method.

Swine of category 2 were assumed to be between 5 and 6 months of age at the time they were killed and averaged about 150 lb. They were unvaccinated and presumably fully susceptible to hog cholera when inoculated with 2 cc. of virulent hog cholera virus six days prior to being killed.

Swine in category 3 were obtained from one source. They were 10 weeks of age and had an average weight of 34 lb. at the time 12 of them were injected with 2 cc. of virulent virus and 30 cc. of hog cholera antiserum. None of these pigs had been given any prior cholera-prevention treatment. Typical symptoms and lesions of hog cholera occurred following inoculation of the pig used to test the virulence of the virus. Daily temperature records were kept of the vaccinated portion of this group and of the virulence test pig. The 6 control pigs were kept together in one pen, separate from those vaccinated. This pen was entered only on the day each pig was to be killed, and the temperature of each of these controls was taken only on the day it was killed.

Collection of Specimens.—The brain from each pig was obtained by removal of the head and sawing the head longitudinally by means of a band saw. One half of the brain was then removed. A portion of the cerebral cortex was obtained from the region of the sylvian and lateral sulci. A portion of cerebellum was taken at its approximate center and included the lateral lobe. A cross section of the brain stem was obtained from the area of the pons. These tissues were fixed for about seven days in 10 per cent formalin and were prepared for histological study. Hematoxylin and phloxine were used in staining.

Bacteriological examinations were made on 88 brains of categories 1 and 3. These examinations were made on the brains of swine killed for autopsy at the laboratory. No bacteriological examination was made of brains from animals that had died prior to the time for necropsy. For the purpose of bacteriological examination the opposite half of the brain used for histopathological examination was removed as nearly aseptically as possible. Portions corresponding to those used for histological examination were removed and placed in sterile glass tubes and retained at −28 C. Later these portions were thawed, seared on the surface, and a small portion removed, placed in 5 per cent tryptose broth and incubated at 37 C. A 3-mm. inoculation loopful of the broth was then smeared across the surface of two types of culture mediums, brilliant green agar (Difco) and 5 per cent swine blood agar. Colonies exhibiting the Salmonella reaction on brilliant green agar and those having the ability to hemolyze swine red blood cells were studied further.

RESULTS

Only inflammatory lesions of the brain were considered in the evaluation of encephalitis; thus precluding any diagnosis on the basis of degenerative lesions not accompanied by inflammation. Perivascular cuffings by "round cells" as described by Hassin,[5] Boyd,[2] and others in nonsuppurative inflammatory infiltrative encephalitides together with foci of such cells not associated with vessels, were sought. Boyd[2] described such infiltrating cells as consisting of small lymphocytes, large mononuclear cells, and occasional polymorphonuclear leukocytes. Meningeal lesions were not considered in this study.

For the purposes of recording the findings with respect to the intensity of brain lesions, an average of all three portions of the brain was used. For each individual portion the following terminology and criteria were used:

O	no lesions found.
+	few vessels exhibiting intravascular aggregations of cells.
++	10 to 50 per cent of vessels exhibiting intravascular or perivascular aggregations of not more than five cells and/or a small number of inflammatory foci of similar magnitude not associated with vessels.
+++	50 per cent or more of vessels exhibiting intravascular or perivascular aggregations of five to ten cells and/or a moderate number of inflammatory foci of similar magnitude not associated with blood vessels.
++++	majority of vessels exhibiting perivascular cuffing of ten to twenty cells and/or numerous inflammatory foci not associated with blood vessels, containing ten to twenty cells per aggregate.

+++++ almost 100 per cent cuffing of vessels and/or multiple inflammatory foci containing twenty or more cells per aggregate.

An average of the degree of encephalitis in each brain was computed from the data of the component parts and recorded as 0, 1, 2, 3, 4, 5. No brain having lesions in at least one part was recorded, in over-all consideration, as less than 1.

Only brains recorded as 3, 4, 5 were considered severely encephalitic.

Category 1 (Naturally Diseased Swine).—Inflammation in some degree was found in 51 of the 100 brains in this category. Thirty-four of the 38 cases diagnosed as hog cholera had lesions of encephalitis. Of the remaining 62 supposedly noncholera cases, 17 were found to have brain lesions. Severe encephalitis was present in 18 of the 34 cholera cases, and in 6 of 17 supposedly noncholera cases.

In addition a more widespread distribution of lesions was found in swine diagnosed as hog cholera. Twenty-five of the 34 swine diagnosed as having hog cholera and any brain lesions had lesions of two or more portions of the brain, as compared to 8 of the 17 of the supposedly noncholera group. Only 14 brains of the 100 examined had lesions in all three portions of the brain, and 12 of these were in the cholera group. Encephalitis was found in 4 of 19 animals vaccinated with hog cholera virus and antiserum seven days or more prior to necropsy and diagnosed as diseased other than hog cholera. The findings in this group are summarized in table 1.

Category 2 (Virus Production Pigs).—In this group, which had been inoculated with virus seven days prior to necropsy, 28 of the 52 brains examined showed some degree of encephalitis. Seven of these were severely encephalitic, none were found to have the maximal degree of encephalitis. Twenty-three had lesions in two or more portions of the brain. Thus, the distribution of lesions was comparable to that found in the naturally occurring cases of hog cholera, but the degree of severity was less. Probably comparison of the two categories is not warranted since the duration of the disease in category 1 was not known.

It was noted that the severity of brain lesions in category 2 was roughly parallel to the gross visceral lesions of cholera in the same animals but probably more striking.

Category 3 (Experimentally Serum-Virus Vaccinated and Controls).—Lesions of encephalitis were found in 4 of the 12 vaccinated and 1 of the 6 controls. The lesions in those vaccinated were found in 1 of each of the pairs killed 8, 13, 18, and 23 days after vaccinations but not in those killed on the third or thirty-first day. None of those vaccinated or controls revealed clinical evidence of encephalitis and none was found to have macroscopic lesions of cholera at necropsy.

Classifications of 3 and 4 were given the brains of 3 of the 4 vaccinated, the fourth was classified as 2. Three of the 4 had lesions in all three portions of the brain, the fourth in two portions.

The pig used to test the virulence of the vaccinating virus showed clinical evidence of disease beginning on the fourth day after inoculation. It died on the fourteenth day and revealed macroscopic lesions of cholera on necropsy. Lesions were found in all three portions of the brain which was

TABLE 1—Summary of Histopathological Examinations of Brains of Naturally Diseased Swine

Diagnosis	No.	No. vacc.*	Any degree of encephalitis	Severe encephalitis**	No. vacc.*/No. encephalitic in any degree
Cholera	38	5	34	18	5/5
Enteritis (nonspecific)	5	2	1	0	1/2
Colitis (nonspecific)	9	1	2	1	0/1
Arthritis (streptococcal)	1	1	1	1	1/1
Arthritis (rheumatoid)	1	1	0	0	0/1
Transmissible gastro-enteritis	3	0	0	0	0/0
Pneumonia (including influenza)	12	4	4	2	0/4
Dysentery	4	3	3	0	1/3
Peritonitis	5	3	1	1	0/3
Rickets	1	0	1	0	0/0
Starvation	2	0	1	0	0/0
Anemia	7	0	0	0	0/0
Hernia	1	0	0	0	0/0
Myocarditis	1	0	0	0	0/0
Injury	2	1	1	0	0/1
No diagnosis	8	3	2	1	1/3
	100	24	51	24	9/24

* Vaccinated with serum-virus seven days or more before necropsy. ** Included in previous column.

given a classification of 2. It was not considered to be severely encephalitic.

The brain of the 1 control, which was encephalitic, was classified as 4. Lesions were found in all three portions of the brain.

Table 2 shows the comparative severity and distribution of brain lesions found in the three categories.

TABLE 2—Comparison of Histopathology of Brains of Groups Examined

Category	No.	Any degree of encephalitis	Severe encephalitis	Lesions in 2 or more portions*	3 portions*
1					
Naturally occurring cholera	38	34	18	25	12
Other naturally occurring diseases	62	17	6	8	2
2					
Hog cholera virus production pigs	52	28	7	22	9
3					
Experimental serum-virus vaccinated	12	4	3	4	3
Controls	6	1	1	1	1
Total	170				

* Portions referred to are cerebrum, cerebellum, and brain stem (*see* text).

BACTERIOLOGICAL EXAMINATION

Bacteriological examination of 88 brains in categories 1 and 3 failed to indicate the presence of Salmonella organisms. Several organisms were isolated which gave the Salmonella type of reaction on brilliant green agar. Further study of these showed they were *Pseudomonas pyocyaneus*. No significance was attributed to these bacteria.

Hemolytic streptococci were isolated from three brains, one of which had lesions of encephalitis. Two of the three isolations were made from pigs which had macroscopic lesions of hog cholera.

DISCUSSION

The lack of serological or other specific tests for the diagnosis of hog cholera has no doubt contributed to confusion and some errors in diagnosis. The classical lesions described by Kernkamp [7] and many others are not always found in individual animals. Animal inoculations are too slow and expensive to be of general practical value.

Therefore, any procedure such as brain examination, which serves as an adjunct to the usual methods of diagnosis, is of value.

The data herein presented indicates that brain examination in itself is not always dependable, although lesions were found in 90 per cent of swine in which hog cholera could be diagnosed by the usual methods. However, lesions of comparable severity were found in 27 per cent of swine presumably affected with diseases other than hog cholera.

It is indicated that vaccination against hog cholera by the serum-virus method may induce encephalitis of sufficient severity to be confused with that of active hog cholera in the period three to thirty-one days following vaccination.

The findings of brain lesions in the experimentally vaccinated group offer a possible explanation of nervous symptoms sometimes noted in herds recently vaccinated by the serum-virus method.

In cholera of six-day duration, encephalitis was not evident in almost half (24 of 52) of the brains examined. Gross lesions of cholera were inconclusive at this stage of the disease. Severe encephalitis was recognizable in only 7 of the 52 pigs. Probably brain lesions develop at a rate comparable with the rate at which gross visceral lesions develop. Therefore, the value of brain examination early in the course of the disease appears limited. The finding of encephalitis in pigs vaccinated by the serum-virus method about a week prior to examination may give little information as to prognosis. The possibility of erroneous diagnosis of hog cholera appears great when microscopic brain lesions are found and gross lesions are not found. When gross lesions are present, the necessity for brain examination is obviated except for academic interest. Encephalitic lesions in pigs suffering from an undiagnosed disease only suggest hog cholera. This finding may be of some value, but does not establish an unquestioned diagnosis of the disease.

Bacteriological findings were considered to be negative. Salmonella were not found in the brain and the hemolytic streptococcal isolations in three of 88 brains examined indicated that these organisms are rarely, if ever, involved in encephalitic disease. Bacteremic salmonellosis, reported to be rather common in the late stages of

hog cholera, was not demonstrated in the brain.

SUMMARY

1) The brains of 171 swine were examined microscopically for lesions of encephalitis.

2) Encephalitis was found in 90 per cent of swine affected with hog cholera under field conditions; 27 per cent of swine affected with other naturally occurring diseases, but presumably free from active hog cholera; 56 per cent of swine experimentally infected with hog cholera for a period of six days; and 33 per cent vaccinated by the serum-virus method between three and thirty-one days earlier, although exhibiting no macroscopic lesions of hog cholera.

3) Bacteriological examination of 88 brains failed to reveal the presence of Salmonella spp. Organisms capable of hemolyzing swine red blood cells were rarely found.

4) The diagnostic application of brain histopathology in swine disease diagnosis is discussed, and a possible explanation of postvaccinal encephalitic symptoms is offered.

Addenda

Since the completion of this work in 1950, two pertinent articles have appeared in the literature. Dunne, *et al.*[13] have described an encephalitic strain of hog cholera virus, characterized by its ability to produce encephalitic symptoms and lesions consistently, its shortened incubation period, and the incomplete protection afforded against it by commercial hog cholera antiserum. Helmboldt and Jungherr[14] have enlarged upon their earlier publication[6] indicating, among other things, that the findings of "encephalitic foci" in vaccinated swine similar to those of spontaneous hog cholera, suggests vaccination failure or inapparent hog cholera infection. They further emphasize that in utilizing disseminated encephalitic lesions for the diagnosis of hog cholera, other known encephalitides in swine should be ruled out. This process requires animal inoculation.

References

[1] Biester, H. E., and Schwarte, L. H.: Listerella Infection in Swine. J.A.V.M.A., *96*, (1940): 339-342.

[2] Boyd, W.: Textbook of Pathology, 4th ed. Lea and Febiger, Philadelphia, Pa. 1943.

[3] Creech, S. L.: *Brucella Suis* Infection of the Brain of Swine. J.A.V.M.A., *89*, (1936): 584-589.

[4] Doyle, L. P.: Posterior Paralysis in Swine. J.A.V.M.A., *90*, (1937): 656-660.

[5] Hassin, G. B.: Histopathology of the Peripheral and Central Nervous Systems. 2nd ed. Paul B. Hocker, Inc., New York, N. Y. 1940.

[6] Helmboldt, C. F., and Jungherr, E. L.: The Neuropathologic Diagnosis of Hog Cholera. Am. J. Vet. Res., *11*, (1950): 41-49.

[7] Kernkamp, H. C. H.: Lesions of Hog Cholera: Their frequency of Occurrence. J.A.V.M.A., *95*, (1939): 159-166.

[8] Kernkamp, H. C. H., and Fenstermacher, R.: Subtypical Hog Cholera. Proc. 51st Ann. Meet. U. S. Livestock San. A., (1947): 96-99.

[9] Ray, J. D.: Pseudorabies (Aujeskys Disease) in Suckling Pigs in the United States. Vet. Med., *38*, (1943): 178-179.

[10] Seifreid, O.: Histological Studies on Hog Cholera. I. Lesions of the Central Nervous System. J. Expti. Med., *53*, (1931): 277-287.

[11] Shahan, M. S., Knudson, R. L., Siebold, H. R., and Dale, C. N.: Aujeskeys Disease (Pseudorabies). North Am. Vet., *28*, (1947): 511-521.

[12] Shope, R. E.: Experiments on the Epidemiology of Pseudorabies. I. Mode of Transmission of the Disease in Swine and their Possible Role in Its Spread to Cattle. J. Expti. Med., *72*, (1935): 101-108.

[13] Dunne, H. W., Smith, E. M., Runnells, R. A., Stafseth, H. J., and Thorp, F.: A Study of an Encephalitic Strain of Hog Cholera Virus. Am. J. Vet. Res., *13*, (1952): 277-289.

[14] Helmboldt, C. F., and Jungherr, E. L.: Further Observations on the Neuropathological Diagnosis of Hog Cholera. Am. J. Vet. Res., *13*, (1952): 309-317.

Pathological Changes of Experimental Vesicular Stomatitis of Swine

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THE PROBLEM of vesicular diseases of swine is no simpler than that of cattle or horses. Cattle are known to be susceptible to foot-and-mouth disease (FMD) and vesicular stomatitis (VS), but not to vesicular exanthema (VE); horses are known to be susceptible to VS and at least to some strains of VE virus, but not to FMD. Swine are highly susceptible to all three of these diseases. The problem of differential diagnosis is ever pertinent. This is especially true at present because of the recent outbreak of FMD in Canada, the spread of VE to many states outside of California where it was localized for twenty years, and the constant threat of VS to the animal industry.

Vesicular stomatitis is an infectious disease characterized by fever and vesicular formation which occurs naturally among cattle, horses, swine, and men. It has been established as a noxious entity since World War I, primarily as a disease of horses and cattle.[1-4] Although it is seldom lethal, VS causes heavy economic loss of work, milk, and meat production of animals. The natural infection among swine was not reported until 1943[5]; it had been shown experimentally, however, that swine were susceptible.[10, 12] The mode of transmission under natural conditions is still obscure; however, some break in the skin or mucous membrane appears to be necessary if the virus is to enter these structures and form vesicles. Heiny observed affected swine in Colorado,[6] but no comprehensive study was made on these swine. The first extensive outbreak of VS among swine in this country occurred in 1943 in a Missouri establishment engaged in the production of hog cholera immune serum.[7] Isolation of the causal virus and subsequent typing revealed it to be the New Jersey type.[8, 9] An outbreak of VS among swine in Colombia was reported in 1946.[11] Apparently, the New Jersey type is the only one that has been detected in natural outbreaks among swine.

According to the available literature, VS has occurred infrequently in swine. There is no doubt they can harbor the virus and serve as a potential source of outbreaks. This paper deals with the pathological characteristics of vesicular stomatitis in swine.

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EXPERIMENTAL PROCEDURE

Seven healthy hogs weighing 120 to 150 lb. were inoculated with vesicular stomatitis virus, New Jersey type (VSV-NJ). The inoculations were by the intravenous (2 hogs), intradermal (3 hogs), or intracerebral routes (2 hogs). The inoculum consisted of allantoic fluid harvested from 9- to 10-day-old embryonating chicken eggs which had died after the introduction of the Wisconsin strain virus at their eighth day of incubation. The titer of the fluid for chicken embryos was $10^4 LD_{50}$. Four of the 7 infected hogs were destroyed at different times, depending upon the clinical appearance of the lesions after exposure in order to obtain various stages of tissue reactions. The remaining 3 animals were permitted to survive in order to observe the entire course of the disease. Sections of various organs were collected from the 4 slaughtered hogs and fixed in Mossman's fixative; the brain tissue was fixed in formalin. The tissues were sectioned and stained with hemotoxylin-eosin and Schorr's stains.

Examinations for clinical signs were made at six-hour intervals during the first few days after exposure, followed by examinations at twelve-hour intervals until recovery occurred. Body temperatures were recorded. The hogs were kept away from other animals and under good husbandry conditions. Blood samples were drawn before the exposures, and serum-neutralization tests for VSV were conducted to detect the possible presence of specific antibody to this virus before the animals were placed on experiment.

RESULTS

The length of the incubation period in swine varied with the route of exposure (fig. 1). Infection by intracerebral inoculation gave the earliest body temperature rise, *i.e.* within twenty-four hours, while intradermal inoculation gave the slowest, *i.e.* seventy-two hours. The intravenous

route of inoculation resulted in a pyrexia in thirty-six hours. The distribution of lesions varied with the routes of inoculation. The lesions produced following intradermal inoculation were localized at the site of inoculation, while intravenous inoculation resulted in the production of lesions at several sites. Following intracerebral inoculation observable lesions were not produced, but there was a thermal response. The first sign of disease was an increase in the body temperature. The body temperature usually ranged between 104 to 107 F.—rarely as high as 108 F. Where vesicles were present, a characteristic drop in body temperature followed rupture of vesicles. Some affected animals went off feed but usually retained their appetite even when, due to the involvement of the feet, they had to stand on their knees to feed. The formation of vesicles of the skin of the lips, snout, the coronary band, and interdigital space was the most characteristic sign of the disease. However, inoculations were not made of the udders or the teats. The lesions of the feet produced in those inoculated intravenously were seen more often than on the skin of the lips and snout and, usually, were the only ones appearing. Sick animals were reluctant to stand and moved with stiffness that suggested the degree of pain. In the case of inoculation in the buccal mucosa, vesicles formed about the lips and the snout but not on the tongue.

The papules usually were not well outlined. They developed into vesicles within a day or two. The vesicles, which were filled with clear fluid, varied in diameter from that of a few millimeters to 3 cm., depending on the location. On the snout, the vesicles were delicate and often of larger size. Once the vesicles ruptured, reddish denuded erosions remained with exfoliated tissue adhering to the margins of the lesion. This was seen characteristically on the snout although quite often it occurred on the coronary band. The erosive stage lasted for a week or so. Although there was swelling of the lips, vesiculation of the snout, and soreness of the feet, the tongue and gums failed to show lesions. The involvement of the coronary area sometimes resulted in loosening and sloughing of the claws. The painfullness of the affected parts was very evident. Animals would resist being touched as early as the papular stage. Secondary infection did not occur. However, it is reported that unsanitary conditions favor the secondary infection. The duration of the sickness was about two weeks if there was no complication. The prognosis was good. No scars remained after healing.

The histopathological changes in the tissues primarily involved the epidermis during the early stages, then extended down to the subcutaneous tissues. The first sign was spongiosis shown by the loosening of epithelial cells (prickle cells) of the Malpighian layer (fig. 2). The intercellular bridges between the prickle cells were stretched in bundles which seemingly were forced apart by the edematous fluid which accumulated intercellularly to form small

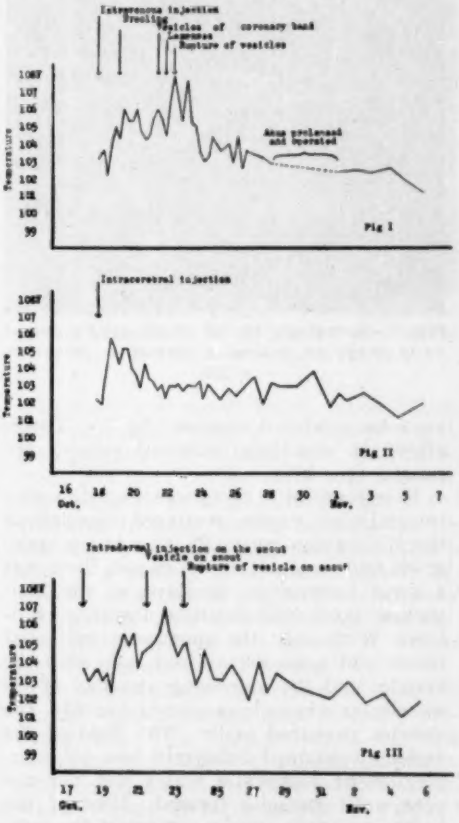

Fig. 1—Correlation of the body temperature response with vesiculation in swine infected with vesicular stomatitis virus.

spaces or vacuoles among the cells. As the process developed, more cells became involved. It was the large number of small vacuoles which gave the gross appearance of the papule. Confluence of neighboring

Fig. 2—Spongiosis of the snout epithelium at early stage of vesicular stomatitis infection. x 350.

vacuoles produced vesicles (fig. 3). Thereafter, the resulting coalesced vesicles appeared as a bleb.

Microscopically, there was a multilocular intercellular edema scattered throughout the Malpighian layer (fig. 4). As the spongiosis and vesiculation developed, there was a great increase in thickness of the Malpighian layer with resulting acanthosis bullosa. With only the spongiotic epithelial tissue and some keratinized cells atop the vesicles and the increasing quantity of intercellular edema immediately beneath, the vesicles ruptured easily. The fluid of the vesicles contained relatively few polymorphonuclear leukocytes which did not appear until vacuoles formed. Most of the leukocytes were caught as part of the infiltrates within the tissues at the base of the vesicle. The epithelial cells after disarrangement by intercellular edema suffered degenerative changes characterized by disappearance of the protoplasmic intercellular bridges and gradual diminution of the cytoplasm. Immediately following such processes the nuclear changes became evident—shrinking, pyknosis and karyolysis—until the nucleus vanished as the cells were being pushed outward.

With these changes, and in addition to vesiculation, the process extended downward into the dermis. The basal cell layer was disarranged and became infiltrated. Papillomatous growth of the basal cell layer was common but was gradually overshadowed by vesiculation and destruction of the basal membrane. Inflammation, including congestion of the dermis sometimes deep into the dermal region, usually was evident in the involvement of the basal membrane. Edema and hemorrhage in the dermal papillae, engorgement of lymph and blood vessels, and perivascular leukocytic infiltration were usually observed. The glandular tissue was not involved. The racemose appearance of the sebaceous glands was retained. Sweat glands were

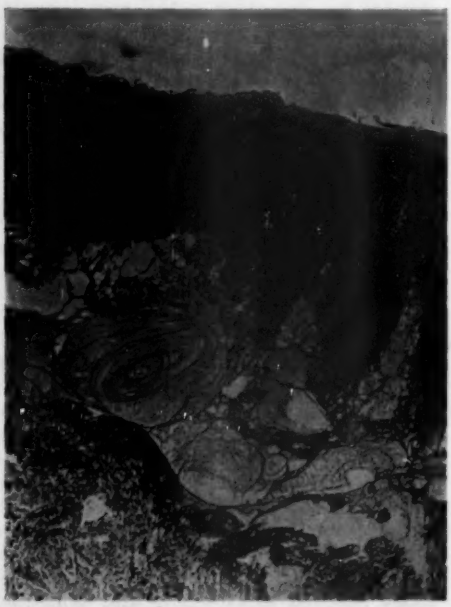

Fig. 3—Vesicle of the Malpighian layer of the skin of coronary band of swine infected with vesicular stomatitis. x 75.

not affected, although sometimes there was some hemorrhage around the hair shaft; however, sycosis and other hypogenesis were not observed. Necrosis at the base of the vesicles was commonly noticed following their rupture.

With the exception of the liver, where congestion was apparent, significant lesions were not seen in other tissues such as the brain, muscles, lungs, heart, spleen, intestines, kidneys, adrenals, pancreas, and lymph glands.

Discussion

The definitive diagnosis of VS is essential because of the constant threat of foot-and-mouth disease. The study of the pathological changes of VS in swine revealed the great similarity of the lesions characteristic of this disease with changes seen in FMD as did a like study of VS in cattle.[14] The same spongiosis and lysogenic degeneration of the epithelial tissue of the skin was seen. However, the mucosal tissue was not affected as in cattle. The same situation often is encountered in VE in swine. Seemingly, the viral action is very specific for prickle cell layer of the skin. The involvement of the dermis is secondary.

The clinical symptoms and histopathological changes serve only to identify VS as a vesicular disease. For an accurate diagnosis, the seasonal incidence, host range, serological distinction, and the location and extent of infections must be employed. The distinct seasonal incidence of VS permits one very largely to rule out VS in outbreaks of vesicular diseases which occur during the cold months. Vesicular stomatitis is prevalent in warm months from late spring to the beginning of freezing weather, while foot-and-month disease and others are not limited in this way. That VS usually disappears after the first frost is shown by all records.[16] Host range is often employed to distinguish these diseases. Vesicular stomatitis has a wide range of susceptible hosts. Eight species of animals have been shown to be susceptible.[17] Among these, the horse is the well-known species that is resistant to FMD, the guinea pig to VE, while both of these are readily susceptible to VS. Serological tests such as the serum-neutralization or complement-fixation tests are comparatively reliable since each of these viruses possesses an individual antigenicity without cross reaction.

The locality of the outbreak also has a certain degree of value. For example, VE has been limited to California in the past[18]; foot-and-mouth infection has not entered

Fig. 4—Multilocular nature of the vesicular stomatitis vesiculation in Malpighian layer of the skin of the coronary band. x 75.

this country in recent years. Nevertheless, the increasing frequency and ease of travel, transportation of animals, and trading of feeds and merchandise cause real concern. The extent of infection differs between the vesicular diseases. During an epizoötic, VS can cover a wide area but rarely are all animals in a herd or in an area clinically affected,[15] as is true of FMD. There are very definite but little understood limiting factors which prevent the spread of VS as seen in foot-and-mouth disease.

To differentiate the vesicular diseases, animal inoculation has been generally recommended. This simple method can fail occasionally to effect a definite diagnosis. The material used for examination should be active and fresh, otherwise it will fail to give accurate results. Chicken embryo inoculation could be employed for VS virus which grows well, while FMD and VE virus

do not. Therefore, for an accurate diagnosis of VS, immunological methods such as serum-neutralization and complement-fixation tests should be employed.

SUMMARY

1) The pathological changes in vesicular stomatitis in swine were studied experimentally with the strain of virus isolated from the outbreak of Wisconsin in 1949.[15]

2) Clinical signs revealed similarity to other vesicular diseases, *i.e.*, foot-and-mouth disease and vesicular exanthema.

3) Histological changes, spongiosis, and vesiculation were similar to those of foot-and-mouth disease.

References

[1] Jacoulet, M.: An sumjet dúne stomatitis erosive de nature indeterminee (Chez le Cheval). Bull. Soc. Cent. Med. Vet., *68*, (1915): 576-578.

[2] Eichhorn, A.: Vesicular Stomatitis. Am. J. Vet. Med., *12*, (1917): 162-170.

[3] Gregg, J., McGuine, F. X., Glover, G. J., Gillespie, A., and Gregory, G.: Vesicular Stomatitis Contagiosa. Am. J. Vet. Med., *12*, (1917): 221-222.

[4] Mohler, J. J.: Vesicular Stomatitis of Horses and Cattle. J.A.V.M.A., *52*, (1918): 410-422.

[5] de Basterreches, Elorrieta L.: La Estomatitis serdo-aftosa o estomatitis vesiculosa en Venezuela y sus diferencias bacteriologicas y clinicas con las glospeda o Fiebre aftosa. Rev. Med. Vet. (Buenos Aires), *24*, (1943): 175-176.

[6] Heiny, E.: Vesicular Stomatitis in Cattle and Horses in Colorado. North Am. Vet., *26*, (1945): 726-730.

[7] Sanders, E. F., and Quinn, A. H.: Vesicular Stomatitis in Swine. North Am. Vet., *25*, (1944): 413-418.

[8] Schoening, H. W., and Crawford, A. B.: Outbreak of Vesicular Stomatitis in Swine and Its Differential Diagnosis from Vesicular Exanthema and Foot-and-Mouth Disease. U.S.D.A. Circ. 734, (1945): 1-14.

[9] Shahan, M. S., Frank, A. H., and Mott, L. O.: Studies of Vesicular Stomatitis with Special Reference to a Virus of Swine Origin. J.A.V.M.A., *108*, (1946): 5-19.

[10] Olitsky, P. K., Traum, J., and Schoening, H. W.: Comparative Studies on Vesicular Stomatitis and Foot-and-Mouth Diseases. J.A.V.M.A., *70*, (1926): 147-167.

[11] Reyer, H. A.: Diagnostic de la cepa EVF como virus de la Estomatitis vesiculosa. Rev. Med. Vet., (Bogota), *15*, (1946): 57-80.

[12] Reppin, K., and Pyl, G.: Foot-and-Mouth Disease or Vesicular Stomatitis. North Am. Vet., *16*, (1935): 19-21.

[13] Traum, J.: Vesicular Exanthema of Swine. J.A.V.M.A., *88*, (1936): 316-327.

[14] Chow, T. L., Hanson, R. P., and McNutt, S. H.: Pathology of Vesicular Stomatitis in Cattle. Proceedings Book, A.V.M.A., (1951): 119-124.

[15] Brandly, C. A., Hanson, R. P., and Chow, T. L.: Vesicular Stomatitis with Particular Reference to the 1949 Wisconsin Epizoötic. Proceedings Book, A.V.M.A., (1951): 61-69.

[16] Hanson, R. P.: The Natural History of Vesicular Stomatitis. Bact. Rev., *16*, (1952): 179-203.

[17] Kowalczyk, T.: The Response of Domestic and Laboratory Animals to Initial and Secondary Exposure to Vesicular Stomatitis Virus. Master of Science Thesis, Univ. of Wisconsin, 1952.

The Histopathology of Nephritis of the Dog. I. Introduction II. Inflammatory Interstitial Diseases

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I. Introduction

DETAILED studies of the pathological anatomy of the dog's kidney are few and incomplete. This may be surprising since renal diseases are extremely common in this species and the dog has been used extensively in the study of kidney physiology and experimental pathology.

Investigators are consistent in their conclusions that lesions of arteriosclerosis are rare in the canine kidney. There is some disagreement, however, as to the incidence of glomerulonephritis. Most of the better documented observations indicate that it is not a common disease. Acute interstitial nephritis, which seldom is recognized in man, is associated with a variety of infectious diseases in the dog, the best known and the most adequately investigated of these being leptospirosis. Little mention is made of pyelonephritis. Contracted, fibrotic kidneys are noted very frequently in dogs but are believed to differ from the primary and secondary contracted kidney of man. Some evidence indicates that they are the result of an interstitial inflammation.

A study of the pathological anatomy of nephritis of the dog should begin with a survey of renal diseases in that species. Material for such a survey was available at the Armed Forces Institute of Pathology because selected tissues and protocols from many excellent cases submitted to the Registry of Veterinary Pathology had been placed in the Institute files. Permission was obtained to examine 395 cases in which distinct lesions were noted in the kidney and, after a careful review, 321 of these cases were selected as most clearly meeting the criteria of nephritis (table 1).

Further studies were not made on the 74 cases of renal disease considered unsuitable for this review because the lesions represented primary metabolic, neoplastic, degenerative, or congenital diseases of the kidney. Cases of true nephrosis were considered primarily a tubular degenerative process and not included in this discussion of nephritis. They can be produced classically in the dog by removal of plasma proteins or by feeding specific toxic agents (mercuric chloride is an example). The 4 cases of nephrosis in this survey were characterized by hemaglobinuria, hematuria, and jaundice and believed to be of an endogenous origin. It is true that most cases of toxic nephrosis develop a distinct tubular nephritis as a result of irritation of tubules. The largest group not included in the 321 cases were the secondary inflammatory changes noted in many of the neoplastic diseases of the kidney; sometimes the concomitant inflammatory reaction was marked.

Hematoxylin and eosin-stained sections from each of the 321 cases were examined and special stains were prepared when necessary for interpretation. It was possible to divide the diseases into two main groups: those in which the inflammation involved primarily the blood and lymph vessels and those in which it was localized in the interstitial tissue.

II. Inflammatory Interstitial Diseases

The grouping of 283 of the total of 321 cases of nephritis as interstitial nephritis is perhaps a higher number than would be encountered in most surveys, since experimental cases from the leptospirosis and filariasis control program instituted at the Front Royal Quartermaster Depot during World War II were included. When the lesions in 83 of the 283 cases were found to more closely approach the criteria of chronic interstitial nephritis and no etiological association of these cases to a recognized disease was evident, an attempt was made to explain the origin of the lesions. Chronic interstitial nephritis, in recent

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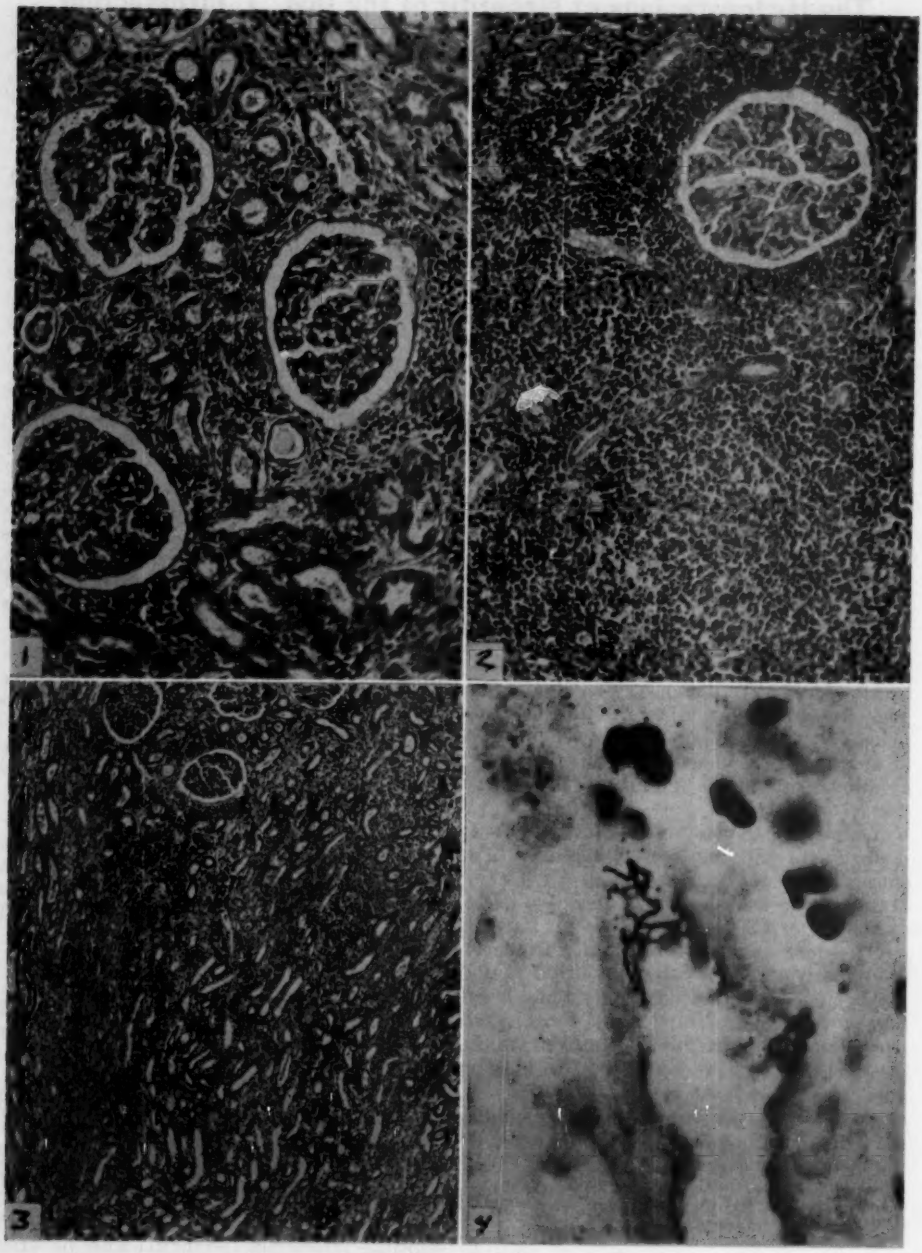

Plate 1

years, has been associated with localization of a not too well-defined group of diseases in the interstitial tissues of the kidney.

Preliminary histological investigations of the lesions of interstitial nephritis described later in this article suggested and supported the possibility of progressive interstitial nephritis. Therefore, the evaluation of 200 cases, not considered chronic, to determine their potentiality for developing in time the changes which are the criteria of chronic interstitial nephritis appeared to be the most rewarding approach. For the purpose of discussion, these 200 cases were divided into three main groups in listing the diagnosis in table 1: (1) recognized diseases which produced typical interstitial nephritis (leptospirosis, filariasis, histoplasmosis, and tuberculosis); (2) nephritis characterized by suppuration but not placed in group 1 because there was no association with a known etiological agent; (3) cases of interstitial nephritis in which definite scarring had not appeared but not identified with groups 1 or 2 and designated in this discussion as acute interstitial nephritis.

TABLE 1—Survey of Renal Diseases of the Dog from Material at the Armed Forces Institute of Pathology, Feb. 1, 1951

Total Cases	395
Cases with a primary disease other than nephritis	74
Cases of nephritis	321
Interstitial nephritis	283
Leptospirosis	79
Filariasis	56
Histoplasmosis	1
Dioctophyme renale	1
Tuberculosis	2
Focal suppurative nephritis	34
Acute interstitial nephritis	27
Chronic interstitial nephritis	83
Inflammatory vascular diseases	38
Acute glomerulonephritis	1
Subacute glomerulonephritis	5
Subclinical glomerulonephritis	2
Chronic glomerulonephritis	1
Sclerosing nephropathy	17
Infarction	12

LEPTOSPIROSIS

Canine leptospirosis has been studied extensively in the United States, following its recognition less than fifteen years ago. While the high mortality in the canine population probably instigated the original investigations, public health considerations have dictated much of the present research.

Although the histopathology of the disease in small groups of dogs had been reported by numerous investigators, it was not until 1945 that a detailed study of a large group was conducted by Jones *et al.*[4] They reported a survey of war dogs which were destroyed following the observation that their serums were positive to the macroscopic agglutination test for leptospirosis. These animals were described as outwardly normal. Tissues from 43 of these dogs were examined microscopically and the subacute interstitial nephritis described in 40 of them was believed typical of leptospirosis. Leptospiral forms were demonstrated in silver-stained sections from the kidneys of 6 of these dogs.

In the present study, leptospiras were demonstrable in silver-stained sections of kidney in 41 cases of suspected leptospirosis. Eight of these cases were from a group

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Plate 1

Fig. 1—Acute leptospirosis (7-year-old male Cocker Spaniel). Lymphocytes, plasma cells, and monocytes are scattered between atrophic and degenerating tubules. Hematoxylin-eosin stain. x 160. AFIP accession 213304.

Fig. 2—Subacute leptospirosis (9-year-old male Airedale). The centers of the cellular masses are composed of histiocytes, large monocytes, and degenerating tubular epithelium. Smaller cells with large nuclei which resemble lymphocytes are seen at the borders of the lesion. Hematoxylin-eosin stain. x 160. AFIP accession 198982.

Fig. 3—Acute leptospirosis (7-year-old male Cocker Spaniel). A diffuse focal cellular infiltration is most prominent at the corticomedullary junction. Hematoxylin-eosin stain. x 48. AFIP accession 213304.

Fig. 4—Chronic leptospirosis (3-year-old male German Shepherd which had a positive agglutination titer for leptospirosis but did not have any noticeable symptoms of a disease). Masses of tightly coiled argentophilic leptospiras with a characteristic hooked end outline the lumen of a tubule. Warthin-Starry stain. x 1100. AFIP accession 175794.

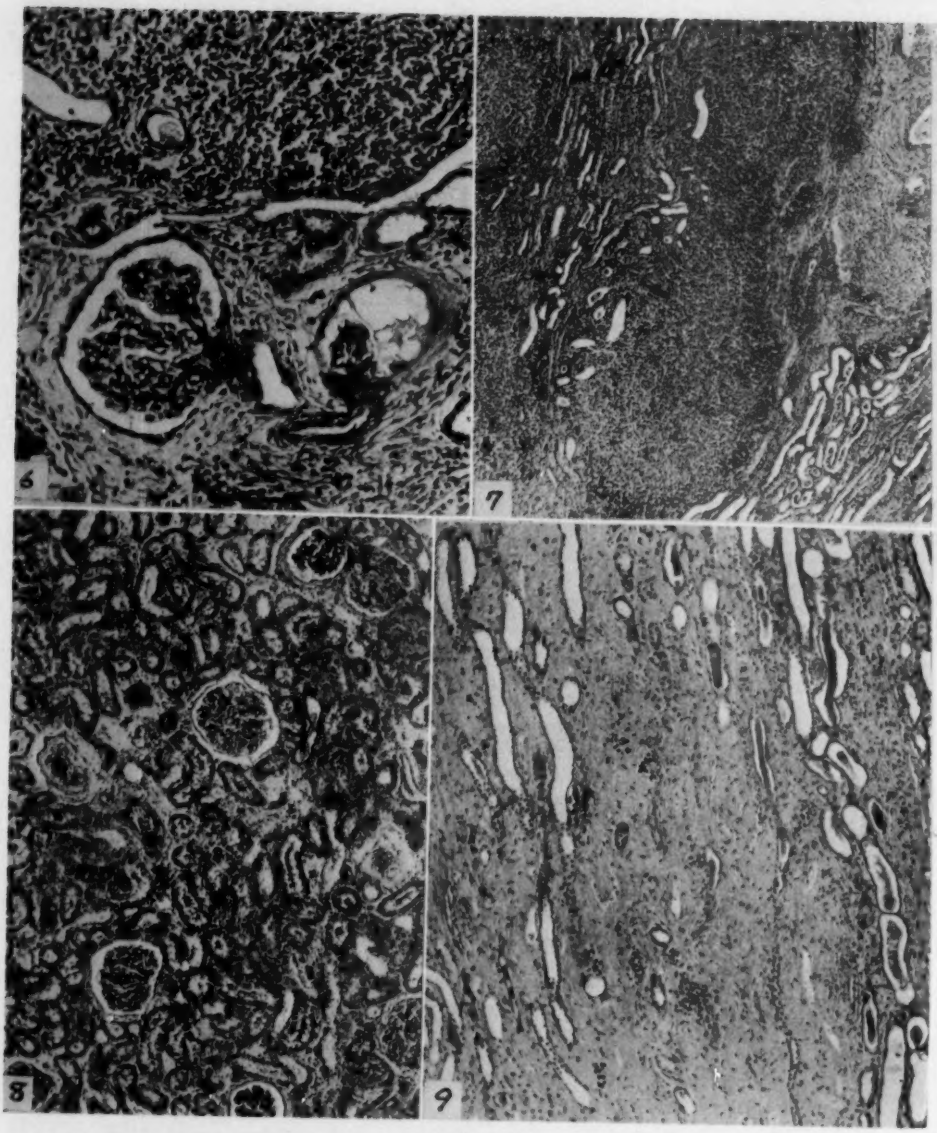

Plate 2

of 46 war dogs which had reacted positively to the macroscopic agglutination test but were not noticeably ill; these 46 war dogs included some but not all of the 43 animals reported by Jones *et al.*[4] The renal lesions noted in 22 of the 41 dogs with demonstrable leptospiras studied in this survey were regarded as characteristic of an acute infection and the remainder as representative of a more chronic stage of leptospirosis.

Monlux,[5] in 1948, in a morphological study of acute leptospirosis in dogs, emphasized that icterus was related to more extensive liver damage and that other lesions of the hemorrhagic type encountered in the kidney were indistinguishable from the icteric type. The acute febrile stage of leptospirosis is the most easily recognized clinically but should not be confused with the terminal uremia of subacute leptospirosis. In our 22 cases of acute leptospirosis, the periglomerular, perivascular, and intertubular infiltrations of lymphocytes, plasma cells, monocytes, reticuloendothelial cells, and a few neutrophils were the most noticeable changes. This infiltration was characteristically more prominent and more extensive at the corticomedullary junction although small infiltrates were sometimes observed throughout the entire cortex in fatal or severe cases (fig. 1 and 3). Aggregates of these cells were seldom seen in the inner medulla and pelvis. Degeneration and atrophy of tubules in the cortex, which have been described by Bloom,[2] were particularly prominent in the proximal convoluted tubules. Many proximal convoluted tubules were desquamated of epithelium or necrotic and in others the epithelial cells had coarsely granular vacuolated cytoplasm and indistinct pyknotic or fragmented nuclei. Epithelial, granular, and hyaline casts were numerous within the tubules which also contained a few calculi. Regenerating tubular epithelium, described by Bloom[2] as occurring in dogs affected with leptospirosis in the third to the tenth day of the clinical disease, was seen in only a few tubules. Tightly coiled argentophilic leptospiras with the characteristic hooked end were demonstrated with appropriate silver stains in the interstitial tissue and tubules of the cortex and were often diffusely scattered in large numbers throughout the parenchyma. They were similar to the organisms represented in figure 4 from a case of chronic

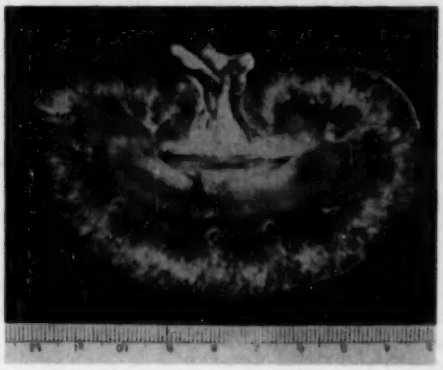

Fig. 5—Subacute leptospirosis (9-year-old male Airedale). Many irregular whitish gray masses are present in the cortex and outer medulla and are most prominent at the corticomedullary junction. AFIP accession 198982.

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Plate 2

Fig. 6—Chronic leptospirosis (3-year-old female German Shepherd). Connective tissue is beginning to appear between glomeruli and tubules adjacent to a nodular lesion of leptospirosis. Hematoxylin-eosin stain. x 160. AFIP accession 133501.

Fig. 7—Subacute leptospirosis (3-year-old male Cocker Spaniel). Large nodular cellular infiltrations with a pale center are seen in the outer medulla. Hematoxylin-eosin stain. x 48. AFIP accession 184078.

Fig. 8—Chronic leptospirosis (3-year-old male German Shepherd). The interstitial cellular exudate is minimal but thickened Bowman's capsules, partially collapsed glomeruli, and a loss of cortical tubules can be seen. Hematoxylin-eosin stain. x 75. AFIP accession 175794.

Fig. 9—Chronic leptospirosis (3-year-old male German Shepherd). The replacement of medullary tubules with connective tissue is focal and there is little cellular exudate in these areas. Hematoxylin-eosin stain. x 75. AFIP accession 175794.

leptospirosis in which leptospiras were found in tangled masses in a small group of tubules. In contrast to the great number of organisms demonstrated in acute leptospirosis, Leptospira organisms were found in only a few isolated tubules in subacute and chronic leptospirosis, and usually it was necessary to make a detailed and not always successful search of specially stained sections in suspected cases. In the acute cases, the kidneys were often described as pale and slightly swollen. Severe congestion of the medulla contrasting with the ischemic cortex was characteristic, and small petechiae were seen in the capsule and parenchyma. Transitional cases from the acute to the subacute stage were unusual.

The subacute stage has been adequately described by Jones *et al.*[4] and Jones [5] who stated that the presenting symptoms resulted from uremia caused by progressive nephritis. The lesions in the kidneys of the subacute cases in this survey in which leptospiras were demonstrable were not distinguishable individually at first from the group of 38 war dogs with positive agglutination titers but in whose tissues leptospiras were not seen. However, when the lesions in the kidneys from these 38 cases reacting to the agglutination test were considered as a group, it was possible to establish histological criteria of a slightly more chronic form of leptospirosis in many which for purposes of discussion will be designated chronic leptospirosis (table 2).

Figure 5 is a kidney representative of the subacute type of nephritis; the dog died 39 days from the date of an acute illness which was typical of leptospirosis. The kidneys in such subacute cases were often slightly atrophic or fibrous on section and had adherent capsules. The most striking lesions were large white or grayish nodular masses in the outer medulla and adjacent cortex which extended as radial streaks throughout the entire cortex of some kidneys. Since this corticomedullary zone also is extensively involved in acute leptospirosis, it could be assumed that there is progressive development of these lesions. Some of the nodular masses were seen microscopically to be composed of mononuclear cells and, in more severe cases, a few polymorphonuclear leukocytes infiltrating between intact but degenerating tubules. In other cases, plasma cells, histiocytes, and cells that perhaps were isolated degenerating tubular and epithelial cells made up the central portion of the masses and were surrounded by smaller cells with large nuclei which resembled lymphocytes (fig. 2 and 7). Since lesions were sometimes minimal in the cases de-

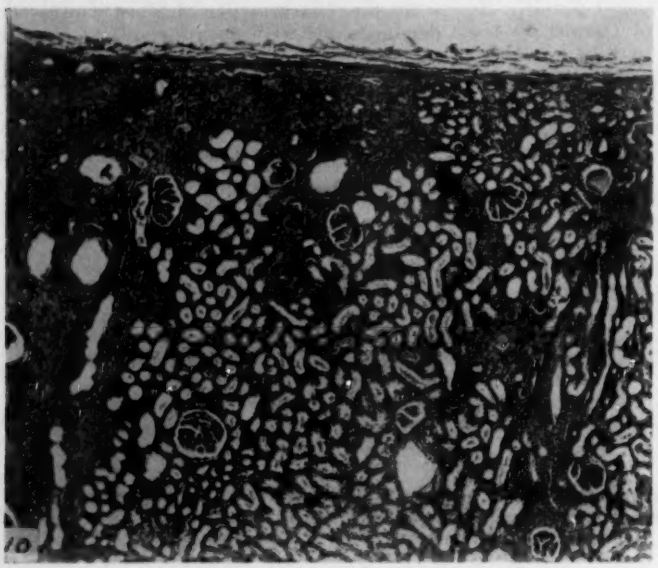

Fig. 10—Focal suppurative nephritis (5-month-old female Boxer). A dense cellular exudate is present in the intertubular spaces and there is a typical linear infiltration along one group of interlobular vessels. Hematoxylin-eosin stain. x 48. AFIP accession 290395.

tected by the agglutination test, it is probable that they were regressing and would not have produced clinically significant symptoms of chronic nephritis.

The presence of connective tissue collagen in the nodular masses of subacute leptospirosis was used as a criterion of a more chronic form of the disease in 23 animals (table 2). This deposition of connective tissue was associated with shrinking of the infiltrations, regeneration and replacement of cortical tubules, and thickening of segments of Bowman's capsule and the glomerular capillaries (fig. 6). In a few cases, the cellular infiltration was minimal, but there were small scars, hyalinized or collapsed glomeruli, and the capsular and glomerular changes already mentioned (fig. 8 and 9). The animal represented in figures 8 and 9 may have been recovering from leptospirosis. The dog was not clinically ill in studies conducted before its death but its agglutination test was positive in the Front Royal leptospirosis control program.

TABLE 2—Variations in Inflammation Noted in Leptospirosis

	Acute	Subacute	Chronic
Cases with symptoms and with demonstrable Leptospira organisms.	22	9	2
Cases without symptoms but with demonstrable leptospiras and a positive agglutination titer.	0	6	2
Cases without symptoms or demonstrable leptospiras but with a positive agglutination titer.	6	19	19

FILARIASIS

Canine filariasis *(Dirofilaria immitis)* is a disease which merits further careful pathological investigation. Schnelle *et al.*[11] and Schnelle and Jones[12] conducted studies on the disease and described gross pathological changes in the heart, lung, bladder, kidney, eye, and other organs. They described the principal gross lesions in the kidney as consisting of scattered inflammatory and hemorrhagic areas in the medulla. These workers also mentioned that the cortex was involved in only a few cases and when affected it was characterized by radial streaking of light-colored tissue. The histopathology of the kidney lesions was not described in these articles.

Tissues from 56 cases of filariasis were examined, 49 from the filariasis control program at Front Royal, Va., and some of these were included in the group reported by Schnelle *et al.*[11] The histological lesions in the kidney varied greatly in scope and intensity. Focal infiltrations of mononuclear cells in the inner medulla were most characteristic. These infiltrations often were marked in the stroma beneath the epithelium of the pelvis. They were particularly noticeable at the borders of the base of renal pyramids where the medulla is narrow and the pelvic epithelium is close to the arciform vessels.* In a few instances, these inflammatory cells were noted in the peripelvic fat at the hilus of the kidney. This pelvic infiltration far exceeded the lymphoid tissue found normally in this area, as verified by comparison with other cases in this study and, in addition, many of the cells were of the plasma cell and histiocyte type. Similar cells occasionally formed large aggregates or linear infiltrations in the medulla or cortex and frequently were arranged perivascularly around the arciform vessels. Necrosis, hemorrhage, and atrophy were sometimes noted in the involved parenchyma, but there was little scarring. Microfilariae were seen occasionally in the capillaries or other blood vessels.

It was known that most of the 49 war dogs in the Front Royal control program had negative agglutination titers for leptospirosis so there was little basis for the assumption that the two diseases were being confused. Furthermore, the medulla and particularly the pelvis were much more extensively involved in filariasis. In leptospirosis, lesions were seldom seen in the pelvis and inner medulla. Kidney infarcts were noted in 2 cases of filariasis and, while no direct relation was proved, it would seem possible that infarcts were due to verminous emboli.

FOCAL SUPPURATIVE NEPHRITIS

If the concept that chronic interstitial nephritis must be preceded by a severe focal inflammation is correct, the only large group of cases which seemed capable of progressing in a few weeks or months to the advanced stage characterized by scar-

* The main medullary mass in the dog's kidney is in the form of a renal crest and indentations of the pelvis around it resemble the calcyces of the human kidney.

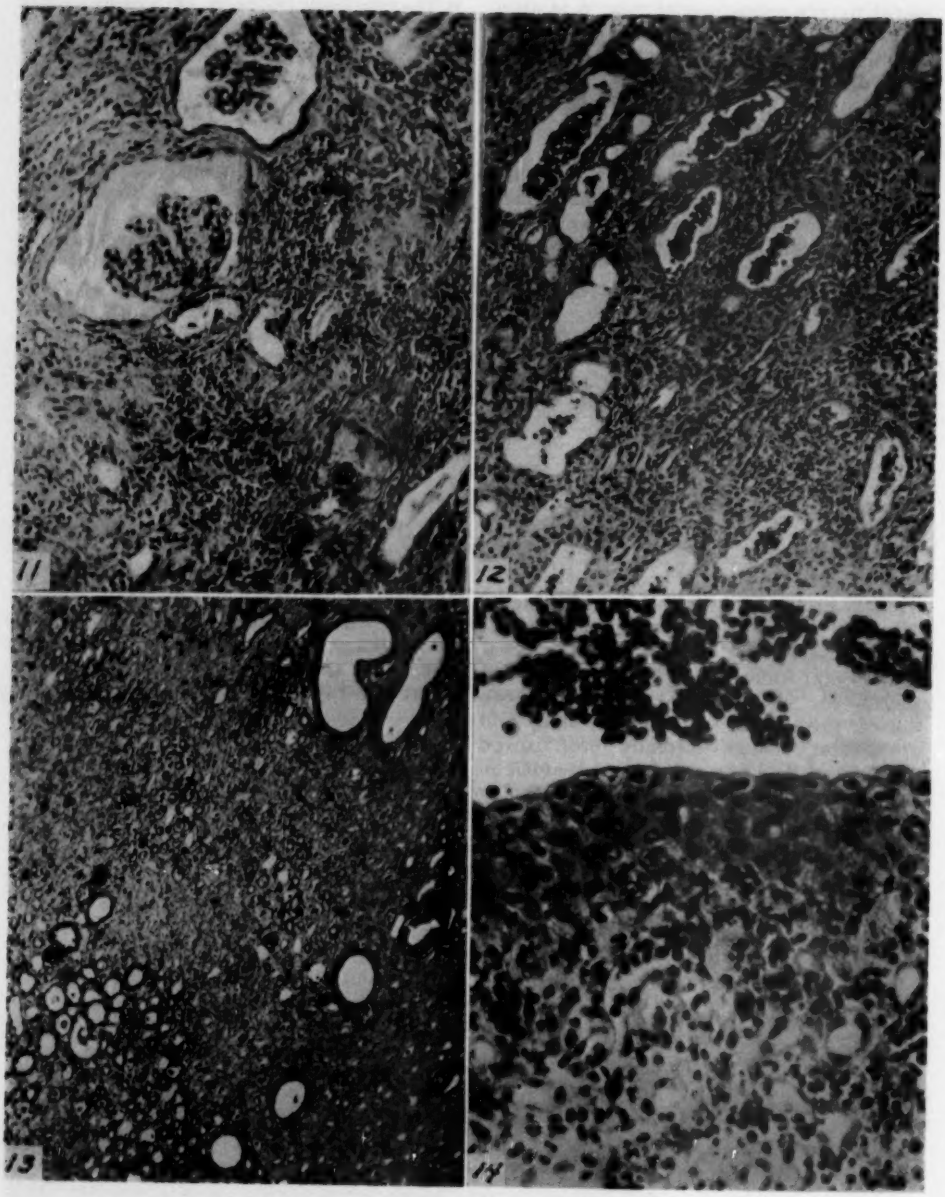

Plate 3

ring and replacement of parenchyma were the 34 cases of focal suppurative nephritis. Suppurative lesions in the kidney usually are associated with specific pyogenic infections which may reach the kidney via the blood stream or by ascending the urinary tract. On the basis of history, autopsy findings, and histological changes, it was concluded that nephritis in only 6 of the 34 cases was the result of an ascending infection. The term "pyelonephritis" as described in human nephritis is too general for classification of the interstitial diseases of the dog and is used only in this study in reference to ascending infections in which pelvis and parenchyma are involved. Inflammatory lesions were noted in the cortex, medulla, and pelvis in all but 3 cases and in these small abscesses were found in the cortex or outer medulla.

These 34 dogs were ill, 16 of them so severely ill that euthanasia had been requested. A nephrectomy was performed on 1 patient from which there is only a surgical specimen. Symptoms compatible with uremia were described in 14 of these dogs. Most of the kidneys were described as swollen with adherent capsules. Occasionally in the same animal, one kidney was slightly contracted and the other enlarged. Irregular yellow or gray areas were seen under the surface of the capsule, and when superficial they could be either raised or depressed. On section linear, circumscribed, or nodular lesions were scattered throughout the parenchyma. Pus was seen in the pelvis in some cases. It was not unusual for the abscesses to involve large segments of the cortex and medulla.

While these partially encapsulated areas were sometimes soft and formed cavities, others were solid masses which resembled the lesions of subacute leptospirosis. When the tissue was examined microscopically, the most striking lesion was the widespread infiltration of polymorphonuclear leukocytes. This cellular exudate was either slightly or completely encapsulated or assumed a linear pattern which filled and distended the intertubular spaces (fig. 10). Necrosis and liquefaction were most prominent in the larger lesions in which the parenchyma was either greatly distorted or had disappeared. Mononuclear cells resembling lymphocytes, plasma cells, and histiocytes were observed at the borders of the lesions or intermixed with the polymorphonuclear cells in the linear infiltrations. The replacement of damaged parenchyma with connective tissue was evident (fig. 11). It was not unusual to see polymorphonuclear leukocytes within tubules (fig. 12). Often the tubules in the involved areas were collapsed or dilated and filled with acidophilic homogeneous casts. Indentations of the capsule in association with the appearance of scars in sections in which the acute lesions were still present (fig. 15) suggested the development of the contracted, pitted kidney. In the medulla there were frequently large areas in which loss of medullary tubules was associated with interstitial infiltration of a finely granular eosinophilic material resembling lymph (fig. 13). Usually only a few polymorphonuclear leukocytes and histiocytes were seen in these areas. The hypertrophy and dilatation of the remaining medullary tubules were proportionate to the degree of medullary damage as evidenced by the loss of tubules. In 2 cases, cellular infiltrations, including neutrophils and lymphocytes,

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Plate 3

Fig. 11—Focal suppurative nephritis (5-month-old female Boxer). The replacement of parenchyma with connective tissue is associated with a distortion of tubules and glomeruli. Hematoxylin-eosin stain. x 160. AFIP accession 290395.

Fig. 12—Focal suppurative nephritis (5-month-old female Boxer). Polymorphonuclear leukocytes are shown within the tubules and scattered within the interstitial tissues. Hematoxylin-eosin stain. x 160. AFIP accession 290395.

Fig. 13—Focal suppurative nephritis (2-year-old male Springer Spaniel). Loss of medullary tubules and a dilatation or hypertrophy of many that remain are seen in areas infiltrated with a finely granular eosinophilic material which is probably lymph. Hematoxylin-eosin stain. x 75. AFIP accession 281926.

Fig. 14—Ascending suppurative pyelonephritis (aged male Collie). Polymorphonuclear leukocytes are present in the pelvis, the pelvic epithelium, and the adjacent interstitial tissue of the medulla. Hematoxylin-eosin stain. x 210. AFIP accession 131848.

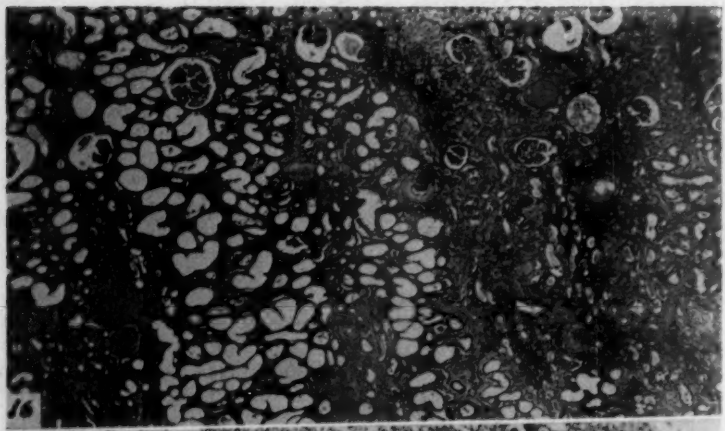

Fig. 16—Chronic interstitial nephritis (5-year-old male Cocker Spaniel). Note the dilated tubules with the thin atrophic, flattened epithelium. Hematoxylin-eosin stain. x 55. AFIP accession 204407.

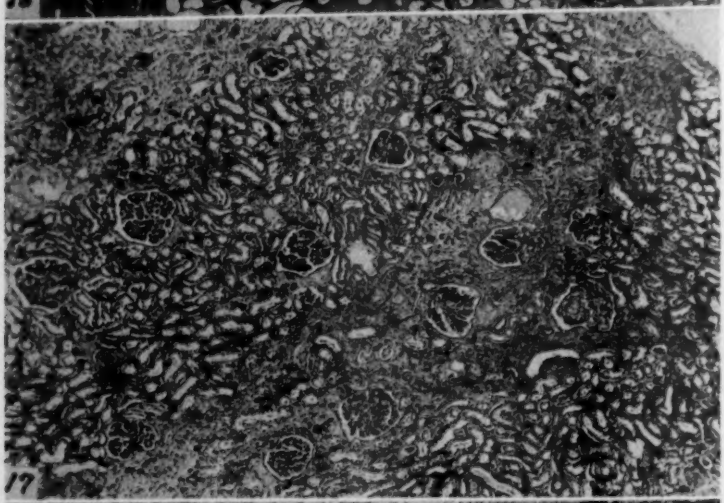

Fig. 17—Chronic interstitial nephritis (12-year-old Airedale). Scars extend irregularly throughout the entire depth of the cortex and separate nephrons which still appear functional. Hematoxylin-eosin stain. x 48. AFIP accession 211523.

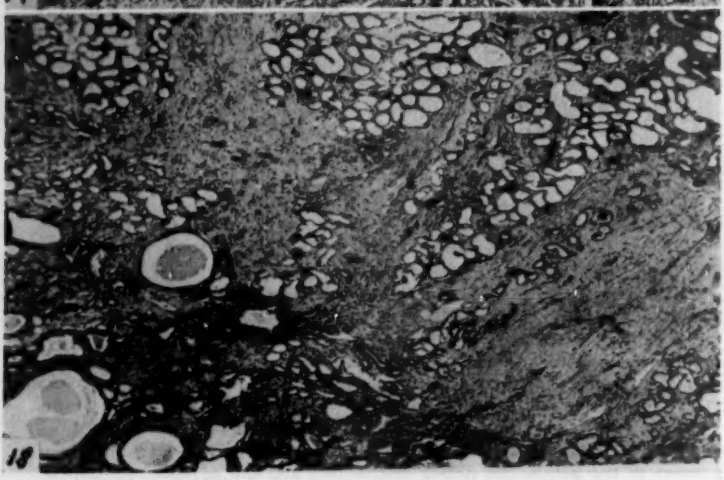

Fig. 18—Chronic interstitial nephritis (9-year-old male Terrier). Large masses of connective tissue are becoming continuous at the junction of the cortex and medulla in this advanced case. Hematoxylin-eosin stain. x 55. AFIP accession 233156.

were present in the perinephric tissues as well as the parenchyma of the kidney. Attempts to identify the more chronic stages of the disease should be aided by the fact that the parenchyma between lesions was altered only by secondary changes in the acute stages. In the discussion of chronic interstitial nephritis, it is pointed out that lesions in less advanced cases are similarly separated by only a slightly distorted parenchyma.

Warthin-Starry stains for Leptospira organisms were prepared in 22 of the 34 cases but argentophilic organisms were not demonstrated. Tissues from ten kidneys were stained by the Brown and Brenn method. In one it was possible to demonstrate gram-positive cocci arranged in long chains in the center of the abscess, and in another gram-positive rods in tubules containing polymorphonuclear leukocytes. In 3 cases, abscesses were found in other organs when all the available tissues on each case were reviewed. These cases of focal suppurative nephritis suggest a definite transition to chronic interstitial nephritis; the beginning fibrosis and the distortion of parenchyma with hypertrophy and dilatation of tubules would progress to lesions indistinguishable from chronic interstitial nephritis.

Ascending Suppurative Pyelonephritis

Six of the 34 cases of focal suppurative nephritis seemed related to an ascending infection. While Bell [1] and Boyd [2] and other writers of textbooks of human pathology emphasize that in man it is possible in most instances to demonstrate obstruction in pyelonephritis, it was not possible to study enough cases to ascertain whether this was true of the dog. However, in 1 case a leiomoyosarcoma of the prostate had produced definite obstruction. The other 5 seemed related to bacterial infections, although microörganisms were demonstrated in association with the lesions in only 1 case when the tissues were stained by the Brown and Brenn method. Two of the 5 cases had large calculi in the renal pelvis which undoubtedly produced partial obstruction. Cystitis, ureteritis, and other inflammatory lesions of the urinary tract were described in these 6 cases. Ascending pyelonephritis in the dog has been described, and there is an excellent case report by Olafson [6] of a *Corynebacterium renale* infection which terminated in pyelonephritis. Histologically, the pelvis and medulla were typically more severely involved in ascending pyelonephritis. It was not unusual to see the pelvic epithelium in-

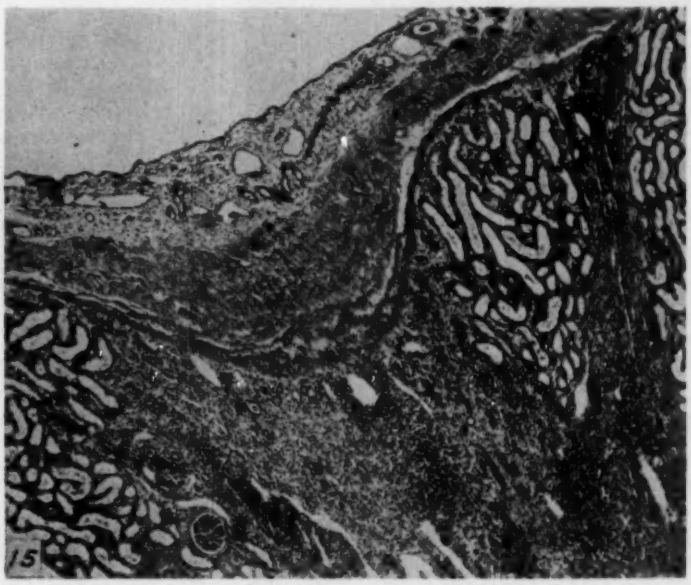

Fig. 15—Focal suppurative nephritis (2-year-old male Springer Spaniel). The indentations of the capsule correspond with the developing scars. Hematoxylin-eosin stain. x 48. AFIP accession 281926.

vaded by polymorphonuclear leukocytes (fig. 14) and the adjacent tubules filled with the same cells

ACUTE INTERSTITIAL NEPHRITIS

The number of cases of acute interstitial nephritis could have been greatly increased in this survey if all the cases of a limited focal interstitial nephritis related to infectious diseases had been included, but this association is so well established that it was not considered necessary to list them. Twenty-seven cases were classified as acute interstitial nephritis. There was no definite scarring, replacement, or distortion of the parenchyma which would allow any of these cases to be grouped as chronic interstitial nephritis.

The nephritis was usually severe and associated with symptoms of acute disease or, in some instances, with uremia. In acute interstitial nephritis, histological examinations revealed many tiny foci of lymphocytes, plasma cells, and mononuclear cells scattered in the inner cortex between the tubules. Polymorphonuclear leukocytes were noted in this cellular exudate but were not prominent. This infiltration was frequently extensive and occasionally involved most of the cortex and outer medulla. It is possible that cases of leptospirosis were included in this group as the lesions were similar, but leptospiras were not demonstrable in sections stained by the Warthin-Starry method.

Fig. 19—Chronic interstitial nephritis (5-year-old male Cocker Spaniel). A typical firm contracted kidney of chronic interstitial nephritis. The surfaces of the kidneys are usually not as uniformly nodular as shown here. AFIP accession 204407.

McFadyean's[6] excellent description of nephritis in dogs in 1929 made no direct association of acute nonsuppurative nephritis with the lesions of chronic interstitial nephritis. However, a few investigators in recent years have postulated, but never actually advanced proof, that acute nonsuppurative nephritis progressed to a subacute stage, and finally to classical chronic interstitial nephritis. The remarkable ability of the kidney to recover from interstitial nephritis is well established in the medical literature. The interstitial nephritis represented by the reaction of the kidney to many systemic diseases should disappear with the recovery of the patient. In the dog's kidney, only suppurative, granulomatous disease and certain parasitic diseases, in addition to leptospirosis and filariasis, could be recognized as producing progressive nephritis with possibilities of developing into chronic interstitial nephritis.

CHRONIC INTERSTITIAL NEPHRITIS

It would be misleading to group cases of chronic interstitial nephritis under this heading and then to discuss only those which were more advanced. In 16 of the 83 cases of chronic interstitial nephritis, the extensive fibrosis and tubular change which usually are regarded as characteristic of the disease were minimal. In the other 67 cases, there was severe nephritis. The kidneys with these more chronic lesions were usually small, firm, and pale, and one kidney in the same animal might be more contracted than the other. The surface was often irregularly or uniformly nodular with some of the nodules so small they appeared granular (fig. 19). In some instances cysts directly beneath the capsule were visible on macroscopic examination. The kidneys were fibrous and cut with resistance. On section, the cortex was narrowed and the normal markings were completely or partially obliterated. The medulla was reduced proportionately and tiny cystic spaces were noted, usually in the outer medulla at the base of the medullary pyramids. Often minute or linear cysts were scattered through the cortex,

but these were usually smaller than those in the medulla.

On histological examination of typical cases of chronic interstitial nephritis, partially fibrosed areas were noted adjacent to parenchyma which was little affected. The contraction of the kidney was related to these depressed scars which were usually not superficial but extended as irregular bands throughout the entire cortex and medulla. The most striking and noticeable changes were in the outer zone of the medulla. In this area, connective tissue and cellular infiltrates surrounded extremely hyperplastic tubules, glandlike in appearance and frequently dilated (fig. 20). These dilated tubules often formed large cystic spaces and their lumina were filled with eosinophilic hyaline casts. The cystic and hyperplastic tubules projected into the cortex and inner medulla as irregular branching extensions in areas bordering on the zones of fibrosis. Other dilated tubules with thin, atrophic, flattened epithelium were intermixed with the hyperplastic tubules and were usually more numerous in the cortex (fig. 16). Partial replacement of the cortical parenchyma by connective tissue resulted in atrophy, collapse, and obliteration of tubules and glomeruli (fig. 17). The Bowman's capsule of the glomerulus often became distended with a homogeneous finely granular material in the zone of fibrosis. As the disease progressed, more and more cystic glomeruli were seen; the tuft of glomerular capillaries became progressively smaller and atrophic as the capsule dilated until the plane of the section included only a cystic space. In a few instances, definite abscesses were noted, but if the advanced lesions were present the cases were grouped as chronic inter-

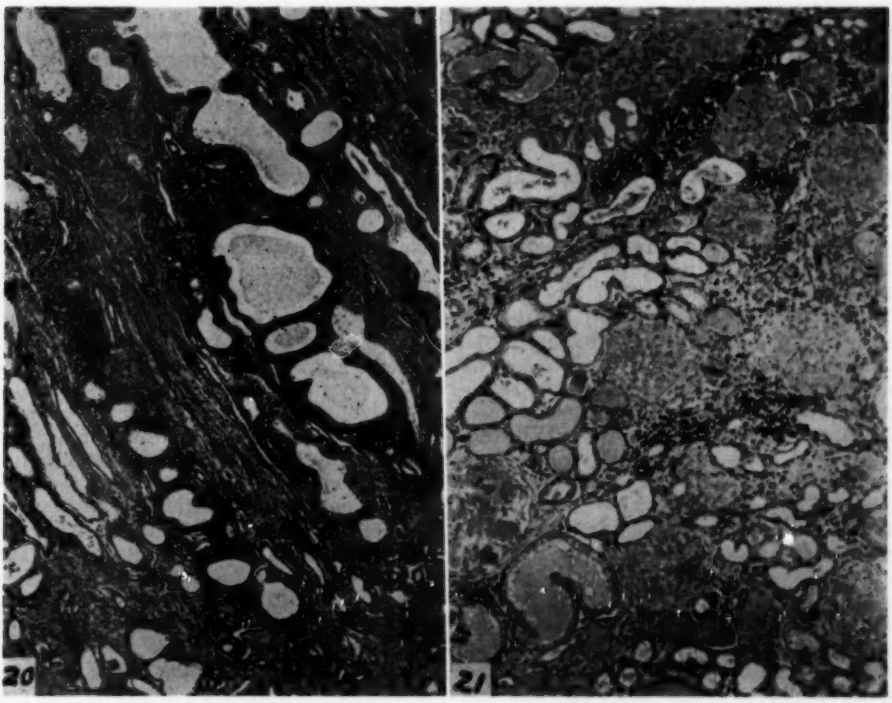

Fig. 20—Chronic interstitial nephritis (4-year-old male Boxer). Hyperplastic tubules are frequently quite dilated in the medulla. Masson stain. x 48. AFIP accession 200570.

Fig. 21—Secondary amyloidosis (5-year-old female Fox Terrier). The cellular infiltration is quite minimal around the deposits of amyloid in the glomerular tufts and the interstitial tissues. Hematoxylin-eosin stain. x 75. AFIP accession 281924.

stitial nephritis. In 1 case, while one kidney had lesions of acute suppurative nephritis, the other was contracted and except for several tiny abscesses was typical of chronic interstitial nephritis. The infiltrates of mononuclear cells, which often were predominantly of lymphocytic type, varied in extent in each case but usually were prominent at the borders of the lesions. The blood vessels were often tortuous in the contracted areas and their walls were noticeably thickened. In the linear lesions in the cortex, the exudative response along the interlobular and intralobular vessels and the severe glomerulitis were associated with thickening and narrowing of the arterioles. In the extremely small contracted kidney, a definite fibrotic band separated the cortex and medulla; it seemed to form in segments which became continuous (fig. 18). Some of the irregularly nodular kidneys had wedge-shaped areas of fibrosis which suggested origin from a healed infaret. It is possible, too, that they could have originated from a slow narrowing of one of the larger renal arteries associated with interstitial nephritis or unrecognized vascular disease.

While this paper is limited to a discussion of the nephritides, it is important to consider the diagnosis of amyloid disease, as it is confused both clinically and histologically with chronic interstitial nephritis. Congo red or crystal violet stains were made in 10 of the series of 83 cases of chronic interstitial nephritis. These 10 cases had prominent hyaline lesions in the glomeruli which were not distinctive in location or appearance. It was not possible to obtain the characteristic tinctorial reaction for amyloid which was noted in 9 cases of amyloid disease studied in the preliminary investigation. The secondary amyloidosis represented in these 9 cases was rather distinctive. Beside the characteristic vascular lesions and deposits of amyloid, the cellular exudate was usually minimal and generally of a lymphocytic character (fig. 21). There were few of the histiocytes, plasma cells, or other larger mononuclear cells which were seen in interstitial nephritis. The scarring was not prominent until the terminal stages.

It is generally accepted that chronic interstitial nephritis represents a group of lesions found in older dogs and in practically all dogs over 8 years of age. However, on the basis of the material examined in the original survey and other histopathological studies, it was noted that many kidneys of older animals were not scarred, and even when scarring was present it could be associated, in some instances, with other diseases. Chronic interstitial nephritis was seen more commonly in dogs over 3 years of age.

The suggestion that leptospirosis may contribute to the development of the lesions of chronic interstitial nephritis has been made frequently in the literature and it is evident that this study attempts to minimize this potentiality. Nevertheless it should be noted that evidence is presented to show that a few cases of leptospirosis develop limited focal lesions of a chronic interstitial nephritis in which scarring is very limited. It was rather surprising and, at first, difficult to accept that the extensive fibrosis and tubular changes of typical chronic interstitial nephritis could not be associated with leptospirosis in this study. The apparent high incidence of this infection (Jones *et al.*,[4] Meyer *et al.*[7]) would warrant further evaluations of the lesions of subacute leptospirosis. Unsuccessful attempts to demonstrate leptospiras with silver stains were made in 48 of the 83 cases of chronic interstitial nephritis. Ten of these dogs, which were 4 years or younger except for 1 which was 5, were contributed by the Veterinary Research Laboratory of the Front Royal Quartermaster Depot and all had negative agglutination tests for leptospirosis within a few months of death. Since Meyer *et al.*[7] and others have stated in their publications that dogs affected with leptospirosis will have positive agglutination titers from two to three years from the date of their initial infection, it would be difficult to associate nephritis with a leptospiral infection in the 10 cases.

The relation of filariasis to chronic interstitial nephritis does not seem important. However, if the recent surveys [10] which indicate that 1 to 5 per cent of the dogs of the United States are infected were accurate, the occasional severe case of interstitial nephritis resulting from filariasis would be encountered in any representative survey.

CONCLUSIONS

Histopathological investigations of tissues from canine necropsies submitted to

the Registry of Veterinary Pathology of the Armed Forces Institute of Pathology confirmed the accepted belief that lesions which most commonly involve the kidney of the dog are those of nephritis.

The nephritis associated with acute and subacute leptospirosis was well defined, but it was difficult to trace the development of the subacute lesions to those of a more chronic nephritis. In the material available for this study, it was much easier to demonstrate Leptospira organisms in the renal tissues in acute than in subacute leptospirosis.

If the concept that chronic interstitial nephritis is preceded by a severe focal interstitial nephritis is true, evidence of progressive transitional changes were only found in an appreciable number of cases in the group of suppurative nephritides. They would explain the pathogenesis of an unknown but an important percentage of the cases. Although slight scarring was noted in a few cases of leptospirosis and filariasis, it was concluded that the development of the advanced lesions of chronic interstitial nephritis was not usually associated with these diseases.

It is apparent that further investigations of the more chronic stages of leptospirosis, filariasis, and suppurative nephritis are needed.

References

[1] Bell, E. T.: Renal Diseases. Lea and Febiger, Philadelphia, 1946.

[2] Bloom, F.: The Histopathology of Canine Leptospirosis. Cornell Vet., *31*, (1941): 266.

[3] Boyd, W. A.: A Textbook of Pathology. 5th ed. Lea and Febiger, Philadelphia, 1947.

[4] Jones, T. C., Roby, T. O., Davis, C. L., and Maurer, F. D.: Control of Leptospirosis in War Dogs. Am. J. Vet. Res., *6*, (1945): 120.

[5] Jones, T. C.: The Significance of Canine Leptospirosis in Military Preventive Medicine. The Military Surgeon, *106*, (1950): 183.

[6] McFadyean, J.: Nephritis in Animals. J. Comp. Path. and Therap., *42*, (1929): 58, 141, 231.

[7] Meyer, K. F., Stewart-Anderson, B., and Eddie, B.: Canine Leptospirosis in the United States. J.A.V.M.A., *95*, (1939): 710.

[8] Monlux, W. S.: The Pathology of Canine Leptospirosis. Cornell Vet., *38*, (1948): 199.

[9] Olafson, P. C.: Pyelonephritis in a Dog Due to *Corynebacterium Renalis*. Cornell Vet., *20*, (1930): 60.

[10] Report on the Special Committee on Filariasis. Illinois State Vet. M. A. North Am. Vet., *26*, (1945): 674.

[11] Schnelle, G. B., Roby, T. O., Young, R. M., and Jones, T. C.: Canine Filariasis: A Study of 100 Cases. North Am. Vet., *26*, (1945): 155.

[12] Schnelle, G. B., and Jones, T. C.: *Dirofilaria Immitis* in the Eye and in an Interdigital Cyst. J.A.V.M.A., *107*, (1945): 14.

The Histopathology of Nephritis in the Dog
III. Inflammatory Vascular Diseases of the Kidney

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THE NEPHRITIDES in which the lesions involved primarily the interstitial tissue have been discussed in section II. In the other cases of nephritis reported in this survey, the more significant lesions were located in the blood and lymph vessels. Table 1 lists the 38 cases of nephritis which were placed in this group. The number was perhaps greater than would be expected and cases were included in which the lesions were limited and in which there had been no clinical record of renal disease. It was believed advantageous to review these less advanced cases in order to present a variety of glomerular lesions which might be confused with a progressive fatal glomerulonephritis and to evaluate the probability that some of the more chronic vascular lesions were originally of a secondary nature and had persisted.

TABLE 1—Survey of Renal Diseases of the Dog from Material at the Armed Forces Institute of Pathology, Feb. 1, 1951

Inflammatory Vascular Diseases	38
Acute glomerulonephritis	1
Subacute glomerulonephritis	5
Subclinical glomerulonephritis	2
Chronic glomerulonephritis	1
Sclerosing nephropathy	17
Infarction	12

ACUTE AND SUBACUTE GLOMERULONEPHRITIS

Acute glomerulonephritis and the closely related subacute form are rare in the canine species. The only established case of acute glomerulonephritis found in this material was that of a 9-year-old male dog which was presented for examination with symptoms of polydypsia, anorexia, and emesis; his temperature at that time was 103.5 F. Because the owner had requested euthanasia of the animal, a clinical investigation was not done. Unfortunately, the case was complicated by generalized malignant lymphoma. However, as shown in figures 1 and 2, there was morphological evidence of acute glomerulonephritis. Practically all of the glomeruli were swollen, apparently nearly avascular, and filled Bowman's capsule. Numerous large endothelial cells and polymorphonuclear leuko-

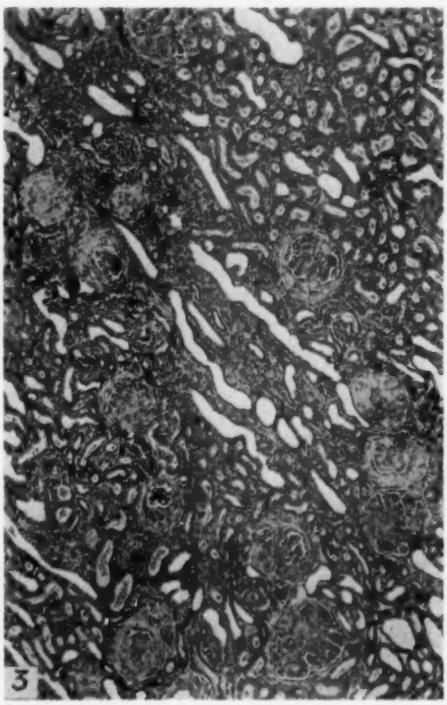

Fig. 3—Subacute glomerulonephritis (1-year-old male Doberman Pinscher). Hyalinization of the epithelial crescents and the collapse and hyalinization of many glomeruli are evident. Hematoxylin-eosin stain. x 75. AFIP accession 266753.

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Excerpts from a thesis submitted in May, 1951, to the faculty of the Graduate Council of the George Washington University, Washington, D. C., in partial satisfaction of the requirements for the degree of doctor of philosophy.

This investigation was supported by a fellowship from the Research Council of the American Veterinary Medical Association and a grant from Parke, Davis and Company. This paper (Part III) was based on material from the Armed Forces Institute of Pathology, as were Parts I and II.

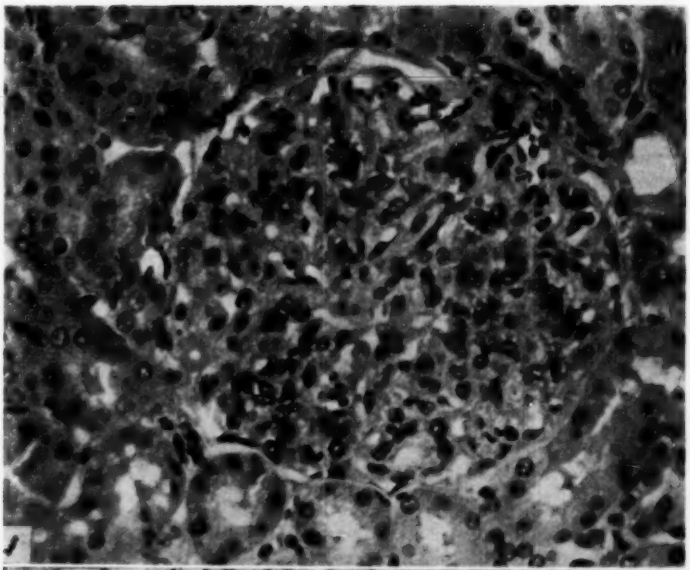

Fig. 1 — Acute glomerulonephritis (9-year-old male mixed Spaniel). Large endothelial cells (arrow) and polymorphonuclear cells protrude into the lumen of many glomerular capillaries. The glomerulus is quite cellular and nearly avascular. Hematoxylin-eosin stain. x 210. AFIP accession 210753.

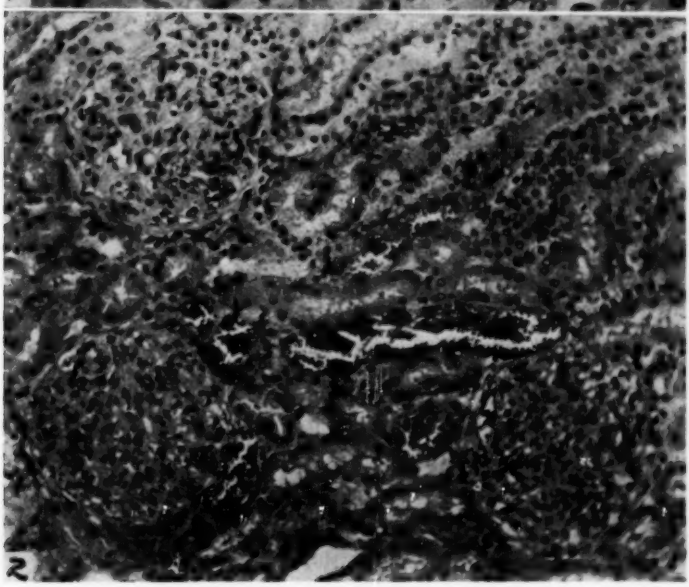

Fig. 2 — Acute glomerulonephritis (9-year-old male mixed Spaniel). Swollen cellular glomeruli almost completely fill the capsular spaces. Hematoxylin-eosin stain. x 185. AFIP accession 210753.

cytes were seen within the lumen of the glomerular capillaries. In addition, neutrophils and monocytic cells not identified as of either endothelial or epithelial origin were often present in what appeared to be a poorly defined edematous interstitial space between the distorted basement membranes of the epithelium and endothelium of the glomerular tufts. Swollen endothelial cells, margination of leukocytes, and inflammatory changes in the walls of the capillaries would seem to be the contributing factors to the ischemia of the glomeruli.

Five cases of subacute glomerulonephritis were studied. This term has been established by Boyd,[2] Bell,[1] and others to designate those cases of acute glomerulonephritis in which the exciting agent is believed to remain after an acute nonfatal stage, so that even though partial healing of the process may take place, there are repeated episodes of illness. After several months, the nephritis terminates in uremia. The case illustrated in figures 3, 4, and 5 (AFIP accession 266753) was typical and well documented as to history, the terminal uremia, and the evidence of nephritis and will not be discussed in detail. Epithelial crescents, hyalinized and occluded capillaries, and associated tubular atrophy were clearly demonstrated. The glomerular lesions seen in figures 4 and 5 were not as advanced as those in figure 3 and the photomicrographs are included to show the pathological variations which were encountered in this progressive form of nephritis. As might be expected, the other

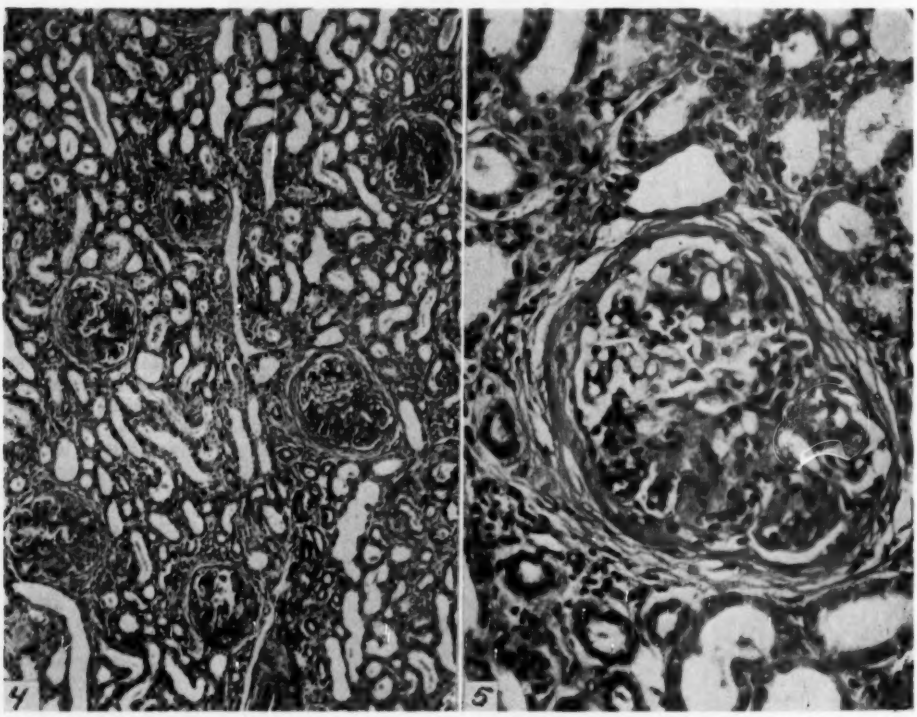

Fig. 4—Subacute glomerulonephritis (1-year-old male Doberman Pinscher). Epithelial crescents can be seen even at a low magnification. Hematoxylin-eosin stain. x 75. AFIP accession 266753.

Fig. 5—Subacute glomerulonephritis (1-year-old male Doberman Pinscher). Higher magnification of one of the glomeruli in figure 4 to show the epithelial crescents, adhesions of the tufts to the capsule, and the partial occlusion of the glomerular capillaries. Hematoxylin-eosin stain. x 275. AFIP accession 266753.

cases were similar but presented slight differences, as is true so often in this disease, which blends with acute glomerulonephritis at one extreme and chronic glomerulonephritis at the other. Glomeruli may be collapsed and the glomerular and tubular spaces filled with an albuminous fluid (fig. 6). One point of interest was that 3 of the 6 dogs afflicted with glomerulonephritis were less than three years of age.

SUBCLINICAL GLOMERULONEPHRITIS

Subclinical glomerulonephritis is difficult to interpret in the dog. Bell [1] has pointed out that in man proliferative glomerulonephritis of varying intensity develops in a notable percentage of infectious and toxic diseases and it is only when capillary obstruction becomes severe that symptoms arise which are recognized as those of acute glomerulonephritis. He further states that glomerulonephritis should be considered an extreme degree of a very common reaction to injury on the part of the glomerular endothelium.

Subclinical glomerulonephritis was used in this paper to indicate those cases of mild persistent nephritis in which distinct glomerular lesions were noted. The suggestion made by many writers that these lesions were secondary toxic manifestations was supported in that a suppurative lymphadenitis was associated with the presence of gram-positive cocci in AFIP accession 261422, and acariasis and filariasis in AFIP accession 209998; the 2 cases illustrated in figures 7 and 8.

Histologically, although there was no infiltration of cells or other evidence of interstitial nephritis, very noticeable thickening and sometimes dilatation of the glomerular capillaries were seen (fig. 7). Endothelial cells were slightly increased in number and noticeably increased in size. However, erythrocytes were still seen in the capillary tufts. Capsular proliferations in relation to the capillary changes were not advanced. Sometimes the glomeruli appeared lobulated, and when the tissues were stained for fat, foci of epithelial proliferation were related to fat deposits (fig. 8).

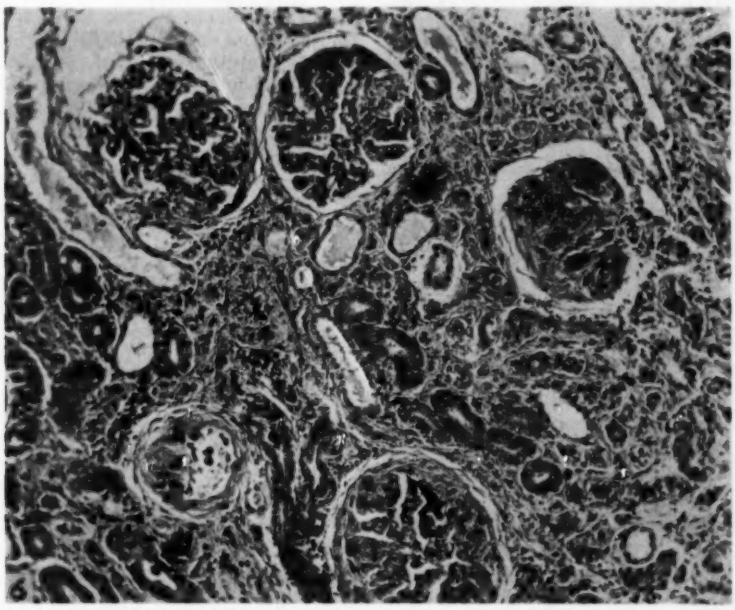

Fig. 6—Subacute glomerulonephritis (2-year-old female Doberman Pinscher). Note the albuminous material in the dilated Bowman's capsule and in many of the tubules. Hematoxylin-eosin stain. x 125. AFIP accession 283398.

SCLEROSING NEPHROPATHY

It was observed in the histological examination of kidneys, which showed little evidence grossly of disease, that many had small, scattered lesions involving Bowman's capsule, the glomerular capillaries, and the juxtaglomerular apparatus. These changes were not extensive enough or sufficiently advanced to justify a diagnosis of glomerulonephritis. They were seldom seen in dogs under 5 years of age. MacNider [3] described a similar capsular and intraglomerular nephropathy in experimental dogs, and it therefore seemed appropriate to designate it sclerosing nephropathy.

A prominent juxtaglomerular apparatus was seen in many of these dogs (fig. 10). In most cases the Bowman's capsule of some of the glomeruli was thickened by proliferation of epithelial cells (fig. 9). Segments of the capillary basement membrane were thickened and in a few instances formed distinct adhesions with Bowman's capsule (fig. 11). The endothelial cells were very prominent but did not appear to be greatly increased in number. Most of the capillaries were merely narrowed and erythrocytes were seen in their lumina. In some instances the juxtaglomerular apparatus was represented by several vascular channels surrounded by a hyalinized mass which was sparsely interspersed with nuclei resembling those of epithelial cells or connective tissue* (fig. 11). The hyaline material between the capillaries did not have the appearance of amyloid when sections were stained with the hematoxylin and eosin stain, and 7 of the more advanced cases were also negative with the crystal violet stain for amyloid. Except for replacement of some of the tubules in the medulla with connective tissue, occasional dilatation of tubules, and a variable infiltration of lymphocytes and plasma cells in damaged areas, there was no other constant lesion in these kidneys.

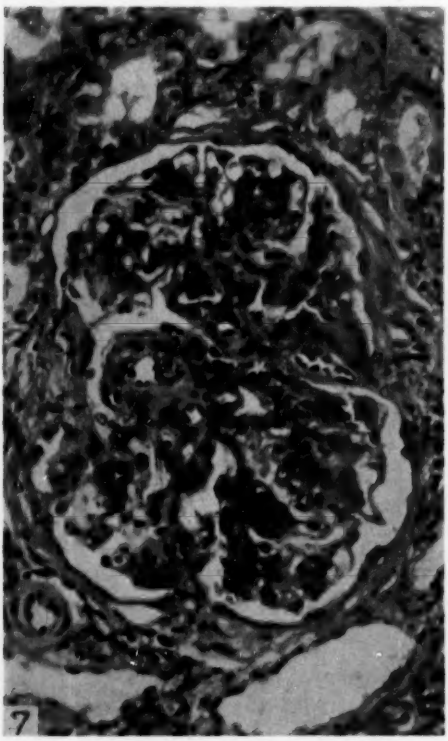

Fig. 7—Subclinical glomerulonephritis (2-year-old male Boxer). Increased cellularity of the glomerular tufts and a thickening of the capillary basement membrane are seen. Hematoxylin-eosin stain. x 275. AFIP accession 261422.

Fat was demonstrated consistently with the oil red O stain in the lesions in the glomerular capillaries and the Bowman's capsule. The fat was very prominent also in the tubules and interstitial tissue of the medulla and was noted occasionally in the epithelium of isolated tubules or in focal deposits in the walls of some blood vessels. It was not birefringent, and it stained blue with the Nile blue sulfate fat stain. The fat was seen additionally in the ascending loop of Henle as in the normal kidney. The presence of fat should not be surprising, for it is associated with many chronic diseases. Fat appeared in subacute or chronic interstitial nephritis in relation to focal lesions but seldom in areas not involved in the inflammation. It was not seen in relation to the inflammatory changes which occurred in acute glomerulonephritis, acute interstitial nephritis, acute leptospirosis, or in lesions which were completely fibrosed or hyalinized. These observations

* It was not possible to make critical identification of endothelial and epithelial cells in most material; the location of the cells, the smaller and more deeply staining nuclei of the endothelial cells and the presence of cytoplasmic fibrils in the epithelial and connective tissue cells were useful criteria.

were supported by fat stains made on 79 cases in this study. It is possible that this widespread distribution of fat in some of these cases of sclerosing nephropathy was related to a preceding interstitial inflammation. Similar glomerular lesions were seen in cases of subacute and chronic interstitial nephritis in which there was a cellular exudate.

INFARCTION

An infarct can be described as an area of necrosis produced by a sudden arrest of circulation. Infarction was included in this discussion of nephritis because of the possibility that it might be associated with inflammatory lesions in blood vessels, particularly those initiated by septic emboli. It was also important to attempt to evaluate the significance and relation of infarcts to the development of chronic nephritis in the dog.

Six of the 12 examples of infarction in this study might have been septic as there were massive infiltrations of polymorphonuclear leukocytes. Endocarditis was demonstrated in 1 of these and also in 2 cases in which the infarct was of the bland type. Four of the 12 had demonstrable infarcts in other organs. An association with a filariasis infection *(Dirofilaria immitis)* was observed in 2 cases of septic infarction.

These infarcts were irregularly wedge or cone shaped with their base near the surface of the kidney. One of the more acute infarcts was congested and classifiable as a red infarct; a narrow zone of only partially necrotic parenchyma directly beneath the capsule suggested a limited col-

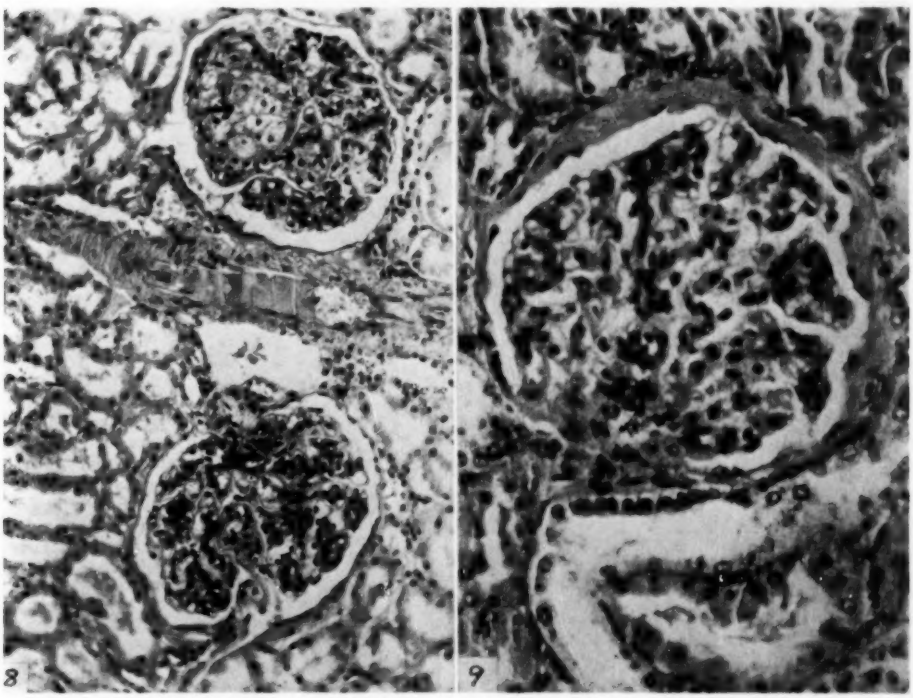

Fig. 8—Subclinical glomerulonephritis (8-year-old male mongrel). Proliferation of epithelial cells (arrow) often give the glomeruli a lobulated appearance. Deposits of fat were found in these cells. Hematoxylin-eosin stain. x 160. AFIP accession 209998.

Fig. 9—Sclerosing nephropathy (a Boxer dog). Bowman's capsule is noticeably thickened. Hematoxylin-eosin stain. x 275. AFIP accession 234712.

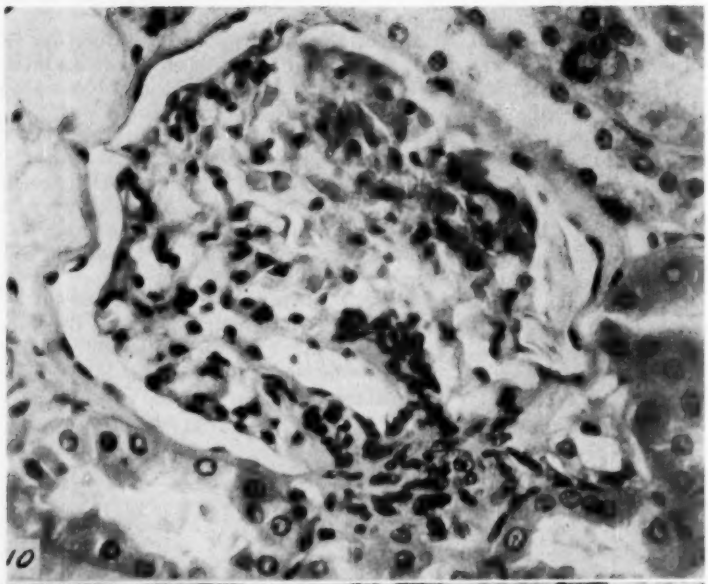

Fig. 10—Sclerosing nephropathy (9-year-old male English Sheepdog). The juxtaglomerular apparatus is enlarged and quite cellular. Hematoxylin-eosin stain. x 435. AFIP accession 187958.

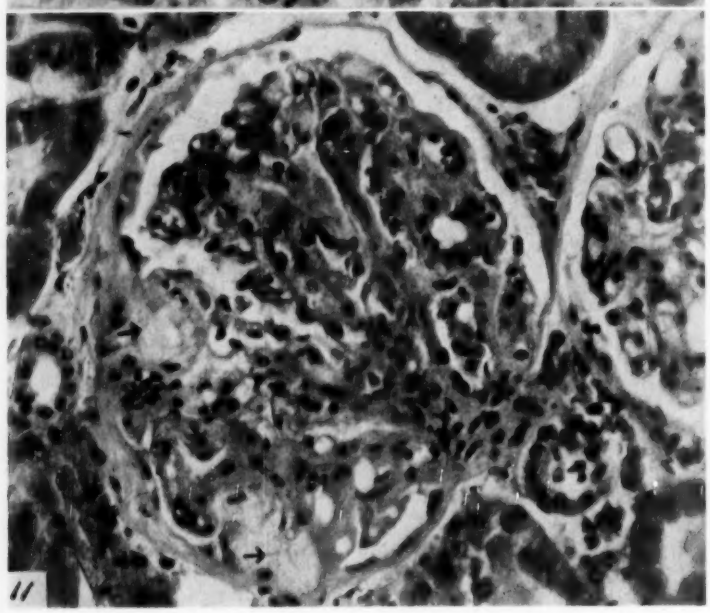

Fig. 11—Sclerosing nephropathy (12-year-old male mongrel). Large quantities of fat were present in the glomeruli in this case and are most prominent in the epithelial proliferations which often form adhesions between the glomerular capillaries and the Bowman's capsule (arrow). Hematoxylin-eosin stain. x 315. AFIP accession 283588.

lateral circulation in that area. However, congestion of the tissue adjacent to the borders of the other infarcts was slight. Basophilic crystalline casts were noted in some of the necrotic tubules in all of the infarcts.

The close relation of the six septic infarcts to suppurative nephritis was evident. In one kidney there were many infarcts and also isolated thrombosed glomeruli associated with infiltrations of polymorphonuclear leukocytes. Connective tissue was present in infarcts in 4 of the cases and would suggest that they were undergoing organization. The possible relation of infarction to chronic interstitial nephritis was discussed in the section on inflammatory interstitial diseases.

CONCLUSIONS

Fatal glomerulonephritis is infrequent in the dog; six cases were found with characteristic histories and lesions.

A variety of lesions involving the glomerulus and Bowman's capsule were described which might be confused with the more advanced changes found in acute and subacute glomerulonephritis.

References

[1] Bell, E. T.: Renal Diseases. Lea and Febiger, Philadelphia, 1946.

[2] Boyd, W. A.: A Textbook of Pathology. 5th ed. Lea and Febiger, Philadelphia, 1947.

[3] MacNider, W.: A Preliminary Paper on the Relation Between the Amount of Stainable Material (Lipoid) in the Renal Epithelium and the Susceptibility of the Kidney to the Toxic Effect of the General Anesthetics. J. Pharmacol. and Exptl. Therap., *17*, (1921): 289.

Glands of the Canine Skin—Morphology and Distribution

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THIS PAPER is concerned with the normal histology of the sweat and sebaceous glands of the dog and their modifications in the ear canal and perianal skin. The study was undertaken preparatory to an investigation of neoplasms of the canine skin glands. Only a few papers in the veterinary, medical, and anatomical literature have dealt with these glands and the chapters in prevailing textbooks on the subject are both scanty and incomplete, particularly with reference to the sweat and perianal glands. It has been a common and erroneous belief that dogs have sudoriferous glands only on the footpads and nose, the remaining integument being without true sweat glands.

MATERIAL AND METHODS

The findings and interpretations are based on biopsy specimens and fresh autopsy materials from over 50 animals of various ages and breeds of both sexes. The tissues have been fixed in Helly's fluid [11] which proved most gentle to the skin specimens. Other fixatives tried tended to make them too hard and brittle. Stains used were hematoxylin eosin, Masson's trichrome, and sudan IV.

For demonstration of sweat, a simplified modification of the iodine-starch technique described by Wada and Takagaki [24] was used. The dry, shaved skin is simply powdered with a dry mixture of pulverized iodine and starch, instead of painting it with water-free solutions of iodine and starch (in absolute alcohol and oil). Sweating was induced in normal animals within five to ten minutes by placing them under an infrared heat lamp, the sweat appearing as small droplets at the base of the hairs. The presence of water in the sweat results in a dark blue stain of the starch, showing as black spots at the openings of the sweat-gland ducts.

OBSERVATIONS

Apocrine Sweat Glands.—Evidence of functional sweat glands in the skin covering the trunk and legs can readily be observed in certain febrile and excited dogs, and in normal animals by exposure to a heat lamp. The sweating was confirmed by the modified iodine-starch technique. Furthermore, this procedure demonstrated that the secretion of sweat occurred in localized patches, probably owing to groups of glands alternating between periods of rest and active secretion.

The sweat glands of the dog's integument, excluding those of the footpads, are all of the apocrine type. They are distributed over the entire skin of the body and extremities, more numerously in some areas than in others. Particularly great

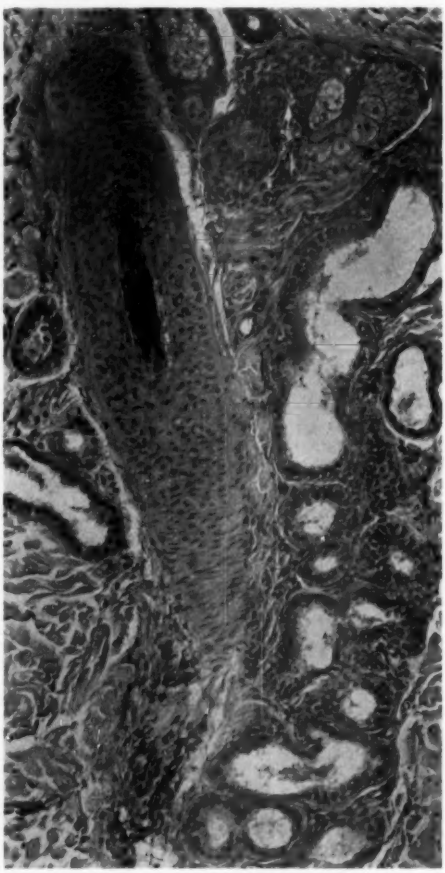

Fig. 1—A section from the skin of the face showing a hair follicle in the fibrous tissue of the corium, surrounded by tubules of apocrine sweat glands. Some sebaceous alveoli are present above the sweat glands towards the skin surface. x 200.

This work was started during the author's residency in Pathology at The Angell Memorial Hospital, Boston, Mass., and finished in the Department of Pathology, Ontario Veterinary College, Guelph, Ontario.

Thanks are due to Drs. Florence Perry and J. P. W. Gilman, Division of Histology, Ontario Veterinary College, for their interest and helpful criticism of this paper.

numbers are observed in the skin on the face and between the toes. The apocrine sweat glands are situated in the dermal and subcutaneous layers of the skin in close connection with the hair follicles (fig. 1). They are, like the apocrine glands in man, situated more deeply in the skin than the corresponding sebaceous glands. Their ducts, however, open into the hair follicles above the ducts of the sebaceous glands.

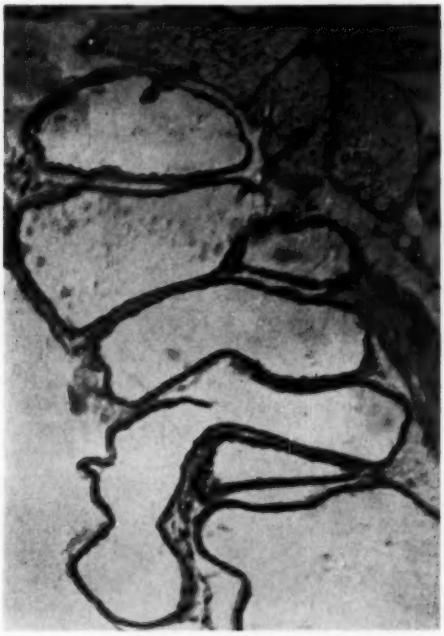

Fig. 2—Dilated apocrine sweat glands from the back of a dog with excessive sweating. Several sebaceous lobules are seen above the cystic, dilated apocrine tubules. The arrector pili muscle of the corresponding hair follicle is seen to the right. x 120.

The apocrine glands are large, loosely coiled glands, with a single layer of secretory cells lining their lumina. A ring of myo-epithelial cells and a fibrous basement membrane are found surrounding the epithelium. The tubules and the individual cells will attain various sizes, depending upon the phase of secretion. In some sections, huge dilated cystlike tubules lined with flattened elongated cells are found (fig. 2), while at the other extreme the tubules are small with high cylindrical epithelium (fig. 3).

The secretory granules accumulate in that part of the cell bordering the lumen, thus forming the apex. These apices vary in size and shape with the secretory cycle. When they have reached their maximum size, they are detached from the basal, nucleated part of the cell, discharge, and dissolve into the lumen. The dilated tubules, lined with a flat epithelium, present the stage immediately following the detachment of the apices and are most numerous in skin sections biopsied from dogs with excessive sweating. The morphological counterparts in the human skin are the apocrine glands connected with the body hairs of the axillary, pubic, and perineal region, and also present on the mammary papillae. They are called odoriferous glands or cutaneous sex glands, because of their specific type of secretion.

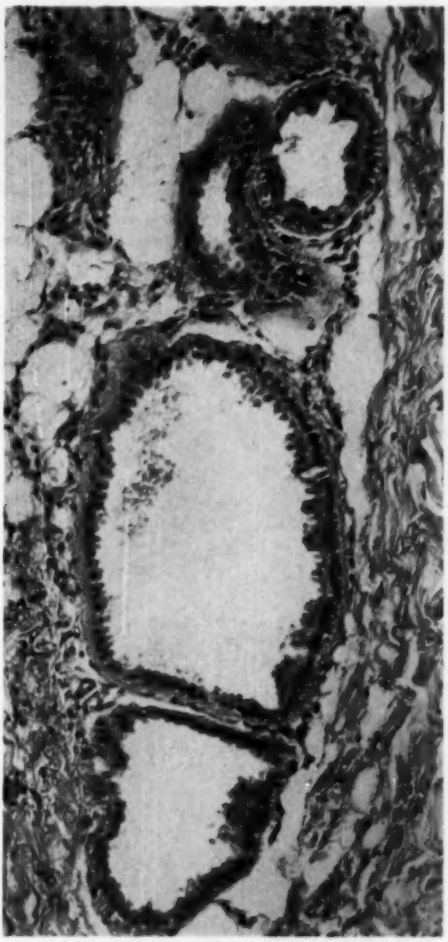

Fig. 3—Section of an apocrine tubule showing the tall columnar cells bearing very distinct apices, protruding as blebs toward the lumina, in which secreta can be seen. x 400.

Fig. 4—Small, coiled merocrine sweat glands situated in the subcutaneous fatty fibrous tissue of the footpads. Note the difference in morphology from the glands pictured in figures 1, 2, and 3. x 800.

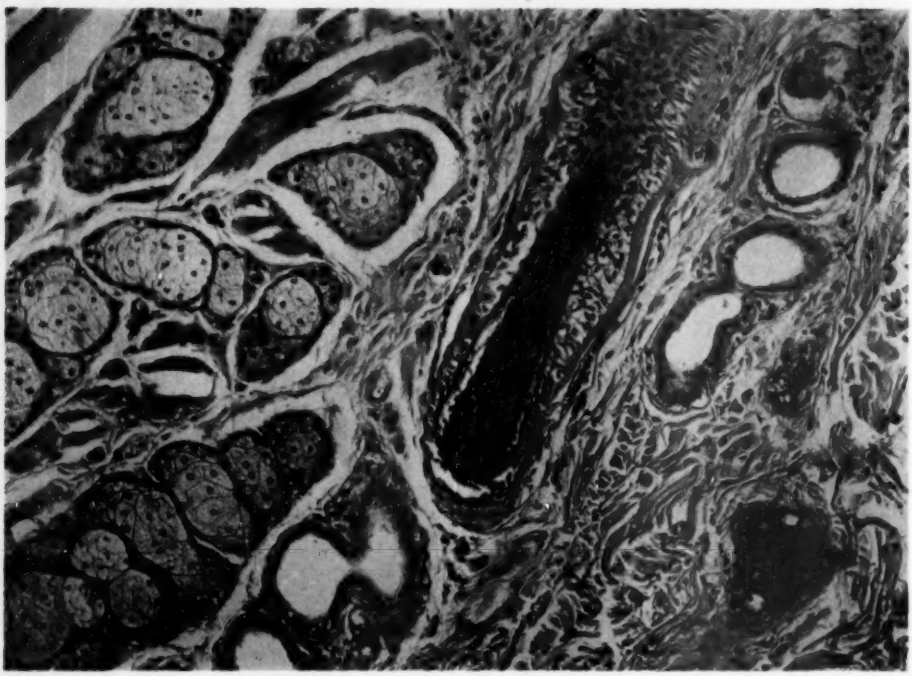

Fig. 5—Corium with the bulbar part of a hair follicle surrounded by apocrine sweat glands and to the left by holocrine sebaceous glands. x 400.

Merocrine Sweat Glands.—Glands apparently having this type of secretion are found on the footpads only (fig. 4). Their histological appearance is similar to that of the sweat glands of the human skin, present in greatest numbers on the

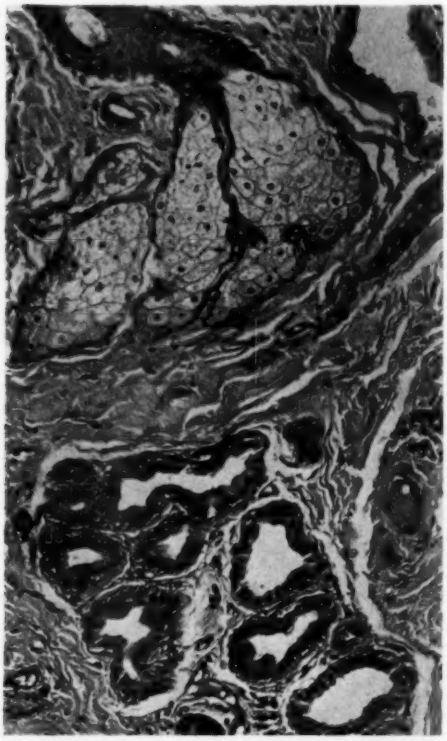

Fig. 6—Ceruminous and sebaceous glands from an ear canal with a chronic otitis externa. The sebaceous glands are found superficially to the apocrine ceruminous glands. x 400.

palms and soles. These are now frequently classified as eccrine because of uncertainty as to their type of secretion. The glands of the dog are placed deeply in the subcutaneous fatty, fibrous tissue under the thick, cornified epidermis of the footpads. They are small, tightly coiled, tubular glands, with minute lumina lined with cuboidal cells, containing rather coarse granules scattered in the clear cytoplasm. Between the secretory cells and the distinct fibrous basement membrane, myoepithelial cells are found at short intervals. The secretory ducts end on the surface of the footpads and their presence is readily seen by placing a dog on a cold table and observing the small drops of condensed water form on the surface. As early as 1835 Gurlt[6] suggested that the glands of the footpads produced the well-known scent of the dog, but this has apparently neither been confirmed nor denied.

Investigations were carried out to determine the origin of the moisture which usually covers the hairless, heavily pigmented, skin of the dog's nose. Serial sections were cut but no glandular tissue was found on the planum nasolabialis, while several tubular glands, both serous and mucous, were found in the fibrous tissue underneath the mucous membrane of the nostrils. Glands on the outside of the nose were found only on the hair-bearing skin, being always associated with hair follicles and, morphologically, belonging to the apocrine sweat glands present in the remaining skin. It seems probable that the droplets of water found on the nose result from a condensation of the moisture blown out from the nasal cavity on expiration.

The Sebaceous Glands.—Glands with a sebaceous, holocrine type of secretion are distributed over the entire integument in connection with the

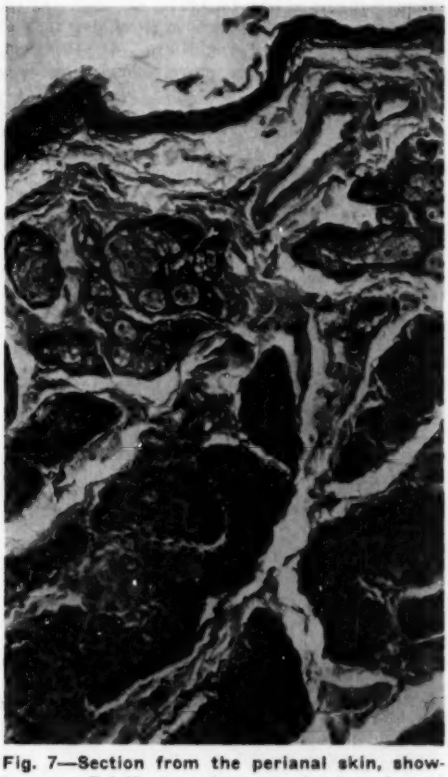

Fig. 7—Section from the perianal skin, showing superficially the sebaceous glands and more deeply the solid lobulated cords of the perianal glands. x 400.

hairs (fig. 5). Morphologically, they do not differ from the sebaceous glands in the skin of other mammalian species.

The Glands of the Ear Canal.—These are apocrine and sebaceous, of similar structure to those of the skin but present in larger numbers. The apocrine glands in the ear canal are called ceruminous glands. The normal secretion, the cerumen, is a product of both glandular types and in a healthy ear canal it appears as a fairly dry, dark brownish substance, differing from the yellow waxy cerumen of the human ear. When hypersecretion and inflammation occur in the canine ear canal, we find histologically a marked hypertrophy and proliferation of both sebaceous and apocrine glands (fig. 6). Whether the infection of the ear canal is the primary lesion with a consequent glandular hypersecretion due to irritation and increased blood supply, or secondary to a hypersecretion with stagnation of secreta, is debatable.

The Peri- or Circumanal Glands.—These are most numerous in the immediate vicinity of the anal orifice (within a radius of 1 to 2 cm.) although they can be found in the skin of the prepuce and groin. This is a particular type of glandular structure developed in the canine species. Microscopically, the glands consist of solid lobules of cells arranged in cords, resembling closely packed liver cells. They are always found in the subcutaneous tissue and do not open into the skin at any point. Above the solid lobules, and in continuity with them, sebaceous glands are present, having their duct openings on the skin surface (fig. 7). The superficial sebaceous glands contain fat, while the deeper solid structures do not. The latter contain cytoplasmic granules possibly of a protein nature. In aged dogs, these solid portions attain striking sizes; cysts and epithelial pearls are found frequently, developing by degeneration and keratinization of centrally situated cells (fig. 8). Neoplastic growth of the peri-

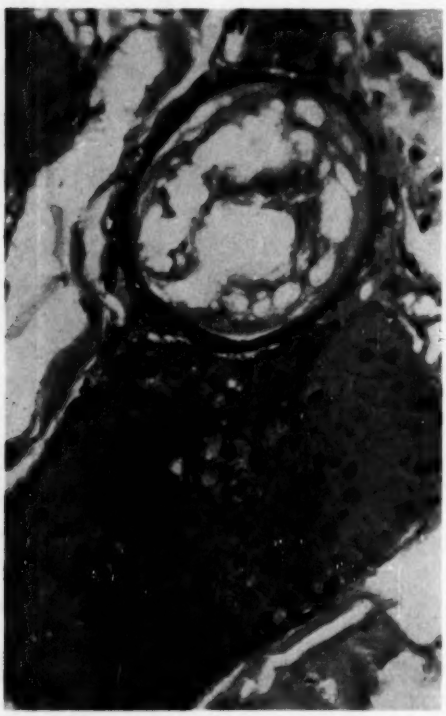

Fig. 8—Perianal gland with cystic dilation of a terminal abortive duct leading from a nonsebaceous lobule. x 300.

Fig. 9—A section from the perianal skin near the anal sac, demonstrating a cluster of apocrine glands of the anal sac. Some striated muscle fibers from the external anal sphincter are seen in the upper left corner. The solid glandular lobules in the lower left are the perianal glands. x 400.

anal glands is a very common finding in old dogs, more frequently in the males than in females.[17, 18]

The Anal-Sac or -Pouch (Sinus Paranalis).— This is a bilateral organ, of pea to walnut size, situated lateroventrally on the margin of the anus, between the internal and external sphincters. It is merely a sacculation of the skin, forming a passive reservoir into which apocrine and sebaceous glands open. The sac is lined with a thin, stratified squamous epithelium, and the underlying loose connective tissue contains many sebaceous and apocrine glands. In general, the sebaceous glands are found close to the neck of the sac, the apocrine being concentrated in the fundus. Morphologically, it is difficult to distinguish the sweat and sebaceous glands of the anal sac from the ordinary cutaneous glands; however, they are present in larger numbers and the individual glands seem bigger than the glands of the hairy skin (fig. 9). Montagna and Parks[18] found that some tubules were actively secretory while others were in a resting phase, a reaction similar to that of the apocrine glands of the skin.

DISCUSSION

It has been commonly believed that dogs can not sweat and that their physical thermoregulation is accomplished by means of hyperventilation or panting rather than by sweating. The observations in this paper on the distribution and morphology of the sweat glands point to the great activity of these glands under certain conditions. That this activity is not the main factor of central thermoregulation was demonstrated by Aoki and Wada.[1] They showed that a dog placed in a heat box will not sweat on the skin areas outside the box, even in the case of excessive rise in rectal temperature and severe panting. They also produced a more localized sweating by means of a heat lamp and demonstrated that this is the result of a peripheral mechanism, since it occurred after removal of the corresponding nerves by sympathectomy. Therefore, they concluded that the sweat glands serve chiefly as a defense mechanism against an excessive local rise in temperature in the normal healthy dog.

Although there seems to be no essential morphological difference in the apocrine sweat glands, the ceruminous glands, and the apocrine glands of the anal sac, the biochemical properties are presumably different.

The perianal glands of the dog have been the subject of much controversial discussion. The findings here support the theory of Parks[19] who examined serial sections, taken at different ages from pups of the same litter, and concluded that the deep solid lobules of the perianal glands are abortive attempts to form sebaceous glands. In the earliest stage of development, the circumanal glands are solid, lobulated structures, the superficial parts of which will undergo sebaceous transformation while the distant portions remain solid. Because of the morphological similarity of these solid portions of liver structure, Schaffer[20] has referred to them as hepatoid glands.

SUMMARY

The distribution of the glands in the canine skin is outlined and their morphology is described.

1) It was found that dogs are capable of sweating and that they have two different types of sweat glands: (a) the large loosely coiled apocrine sweat glands distributed over the entire skin as an appendix to the hair follicles; (b) the small, densely coiled merocrine sweat glands present only in the thick fatty fibrous tissue under the footpads.

2) The hairless, heavily pigmented skin on the tip of the nose was found to be without sweat glands. The moisture usually found here is probably from the glands of the mucous membranes of the nostrils.

3) The ear canal is lined with sebaceous and ceruminous glands, the latter being of an apocrine type similar to the large sweat glands of the skin.

4) The perianal glands consist of cords of solid lobules subjacent to a layer of sebaceous glands. They appear to be nonfunctional and are considered abortive attempts to form sebaceous glands.

5) The anal sac is merely an invagination of the skin, into which sebaceous and apocrine glands secrete.

References

[1] Aoki, Tsuyoshi, and Wada, Masao: Functional Activity of the Sweat Glands in the Hairy Skin of the Dog. Science, *114*, (1951): 123.

[2] Cowdry, E. V.: Textbook of Histology. 3rd ed. Lea and Febiger, Philadelphia, (1944): 385.

[3] Bailey: Textbook of Histology. 9th ed. W. Wood and Co., Baltimore, (1936): 353.

[4] Bremer, Z., and Weatherford, H.: Textbook of Histology. 6th ed. The Blakiston Co., Philadelphia, (1948): 568.

[5] Dahlgren, U., and Kepner, W.: Principles of Animal Histology. 1st ed. The MacMillan Co., New York, (1930): 398.

[6] Gurlt: Vergleichenden Untersuchungen über die

Haut des Menschen und der Haüs-Saugetiere, besonders in Beziehung auf die Absonderungsorgane des Haut-Talges und des Schweisses. Arch. f. Anat., Physiol. u. wissensch. Med., (1835): 399.

[7] Ham, A. W.: Histology. 1st ed. Lippincott Co., Philadelphia, (1950): 155.

[8] Jordan, H. E.: Textbook of Histology. 8th ed. Appleton-Century Co., New York, (1940): 267.

[9] Kendall, J. I.: Microscopic Anatomy of Vertebrates. 3rd ed. Lea and Febiger, Philadelphia, (1947): 165.

[10] MacLeod, J., and Muende, I.: Practical Handbook of the Pathology of the Skin. 3rd ed. P. B. Hoeber, London, 1948.

[11] Mallory, F. B.: Pathological Technique. W. B. Saunders Co., Philadelphia, 1942.

[12] Maximow, A., and Bloom, W.: A Textbook of Histology. 6th ed. W. B. Saunders Co., Philadelphia, 1952.

[13] McClelland, R. B.: The Pathology of the Apocrine Sweat Glands in Dogs. Paper, Medical Society of the State of New York, April 29, 1941.

[14] Montagna, W., and Parks, W.: A Histochemical Study of the Glands of the Anal Sac of the Dog. Anat. Rec., *100*, (1948): 297.

[15] Montagna, W.: The Glands in the External Auditory Meatus of the Cat. J. Morphol., *85*, (1949): 423.

[16] Montagna, W., Noback, C., and Zak, F.: Pigment Lipids, and Other Substances of the Glands of the External Auditory Meatus of Man. Am. J. Anat., *83*, (1948): 409.

[17] Mulligan, R. M.: Neoplasms of the Dog. Williams and Wilkins Co., Baltimore, 1949.

[18] Nielsen, S. W.: Unpublished data.

[19] Parks, H.: Morphological and Cytochemical Observations on the Circumanal Glands of Dogs. Doctor of Philosophy Thesis, Cornell University, Sept. 1950.

[20] Schaffer, J.: Drüsen von einem bisher unbekannten (hepatoiden) Typhus. Wein, Math. Natur. Kl. Anzeiger, *60*, (1923): 37 (quoted by Parks).

[21] Siedamgrotsky, O. A.: Uber die in einigen Haustiere vorkommenden Drüsen. Arch. Wissensch. prakt. Tierheilk. *1*, (1875): 438 (Quoted by Parks).

[22] Trautmann, A., and Fiebiger, J.: Lehrbuch der Histologie und Vergleichenden Microskopischen Anatomie der Haustiere. Paul Parey, Berlin, 1947.

[23] Trautmann, A., and Fiebiger, J.: Fundamentals of the Histology of Domestic Animals. Translation and Revision by R. E. Habel and E. L. Biberstein. Comstock Publishing Co., Ithaca, 1952.

[24] Wada, M., and Takagaki, T.: On Iodine-Starch Method. Tohoku J. Exptl. Med., *49*, (1948): 284.

Studies in Avian Neoplasia. IV. Natural Transmission of Tumor Agents Through the Egg

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THE EGG has been considered as a source of neoplastic infection for some time.[4, 10-13] More recently, Cottral[3] has referred to evidence which indicates that the agent causing lymphomatosis may be present in eggs laid by certain apparently normal hens. The report of Cole and Hutt,[2] however, suggested that there was no evidence for the egg carrying the agent which causes leukosis.

In an attempt to study the relationship of the egg to tumor-agent transmission, the presence of lymphomatosis and Rous-neutralizing antibodies (RNA) was determined in certain avian populations hatched with chicks from infected sources, whose histories had been definitely established. The prevalence of lymphomatosis was also determined in a tumor-infected and in a relatively tumor-free flock as well as in the offspring from these populations which were hatched and raised separately under strict isolation.

METHODS

The following types of avian populations were studied:

1) Two groups of chicks were hatched and raised together: RPL-1 * from a flock with a 41 per cent prevalence of lymphomatosis, and the other VRI-1 ** from stock with a 3.8 per cent prevalence of this disease. A third group, VRI-C, from the same trapnest hens as the VRI-1 chicks, were hatched separately and raised in isolation as unexposed controls. Fifty per cent of the RPL-1 population was inoculated with an attenuated Rous-virus vaccine † in an attempt to alter the prevalence of lymphomatosis. About equal numbers of the RPL-1 and VRI-1 chickens were placed in individual brooder houses. Unvaccinated isolated controls consisting of RPL-1 and VRI-1 birds were placed in an individual brooder house at the time the other birds were vaccinated. Losses of 20 and 3 per cent were experienced in the RPL-1 and VRI-1 birds, respectively, due to perosis, Newcastle disease, coccidiosis, and cannibalism. Serums for the Rous neutralization test (RNT) were collected from RPL-1 birds at 2 months of age and then at monthly intervals for eighteen months. The VRI-1 birds were bled at 7, 12, and 18 months of age. Serums were collected from the unexposed Group VRI-C controls at 1, 2, 8, and 18 months of age. The presence of RNA was determined through a procedure described by Duran-Reynals[5, 6] and modified by Kenzy.[8]

2) A year later, offspring (RPL-2) from the RPL-1 flock were hatched and raised with VRI-2 chicks. Control VRI-2 chicks, sibs of the exposed VRI-2 chicks, were hatched separately and raised in a different brooder house.

3) Eggs from a flock of (BB-E ‡), White Leghorn-Rhode Island Red cross, with a 37 per cent loss due to lymphomatosis, were hatched with RIR § and VRI-3 eggs. Control RIR chicks, sibs of exposed RIR chicks and VRI-3 chicks, sibs of exposed VRI-3 chicks, were hatched and raised separately in individual brooder houses.

Caretakers for the flocks, described in paragraphs 1, 2, and 3, also cared for the control laying flock * from which the VRI chicks for these studies were obtained. Feeding and watering utensils were not interchanged, and the cleaning equipment was disinfected before use in each of the various brooder houses.

4) Eggs from a White Leghorn flock (RTF-1) with an 8 per cent prevalence of lymphomatosis (over a twelve-month observation period) were hatched in a new incubator which was disinfected with formalin and placed in a new, disinfected isolation room completely separated from all other birds. These RTF-2 chicks were placed on litter and raised in this room under strict quarantine. Caretakers always wore freshly laundered coveralls and caps which had not been used in any other poultry building. Boots or rubbers were scrubbed with a detergent-lye solution. Separate equipment was used for cleaning, feeding, and watering.

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The coöperation of the Veterinary Research Institute staff is gratefully acknowledged.

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* Obtained as trapnest eggs, which were pedigree-hatched, from line 9 of the Regional Poultry Research Laboratory, East Lansing, Mich., through Mr. Berley Winton, director, and Dr. N. F. Waters, geneticist. A history of 41 per cent loss in this line due to lymphomatosis over a two-year period was also made available.

** Pedigree-hatched chicks from inbred White Leghorn families maintained at the Veterinary Research Institute.

† Treated with formalin so that 0.2 ml., given subcutaneously, failed to produce tumors in 2-week-old chicks. No neutralizing antibodies were demonstrated in serums from 3-month-old test birds bled three and eight weeks after vaccination.

‡ All these birds had definite levels of RNA in their serums during the six-month observation period before eggs were collected for hatching. The male, which had at least 79,000 Rous-neutralizing doses per milliliter of serum, was obtained from a White Leghorn flock with a history of 48 per cent lymphomatosis.

§ Rhode Island Red egg source with a 5 per cent prevalence of lymphomatosis.

* White Leghorns with a 3.8 per cent prevalence of lymphomatosis.

5) Eggs from flock BB-W ** with a 37 per cent loss due to lymphomatosis were hatched and raised under the same conditions as the birds from the RTF-1 eggs. However, in this case the caretaker entered the BB-W1 room after caring for the RTF-2 birds without changing clothes or disinfecting boots.

All the dead birds from the above populations were autopsied and examined for pathological changes. Tissue examinations were carried out if necessary to complete a diagnosis. The identification and classification of neoplastic changes were based on Feldman and Olson's discussion of avian neoplastic diseases.[7] In the case of the RPL-1 and VRI-1 populations, the survivors were also autopsied and microscopic tissue examinations were usually carried out.

RESULTS AND DISCUSSION

When VRI-1 eggs from a flock with 3.8 per cent loss due to lymphomatosis were hatched and raised with chicks from RPL-1 eggs, selected from a flock with 41 per cent lymphomatosis, the prevalence of this disease in the VRI-1 chickens increased to 29.3 per cent (table 1). Since the VRI-1 birds were exposed to only RPL-1 eggs and birds, it appears that the tumor infection was introduced with the RPL-1 eggs. Similarly, a year later when RPL-2 eggs were hatched with VRI-2 † eggs, lymphomatosis was observed in 40.5 per cent of the exposed VRI-2 birds as compared to no lymphomatosis in the VRI-2 controls during an observation period of one year (table 1). The lack of observed lymphomatosis in the RPL-2 birds, and the high prevalence of this disease in the exposed VRI-2 birds, would suggest that the RPL-2 birds were latently infected with the lymphomatosis virus (table 1).

TABLE 1—Transmission Studies in Avian Lymphomatosis

Population studied	Year	Lymphomatosis (%) and cases observed	Observation period
Control VRI-1	1950	3.8 (19/500) *	2 years
RPL-1		14.4 (15/101)	2 years
Exposed VRI-1		29.3 (27/92)	2 years
Control VRI-2	1951	0.0 (0/32)	1 year
Exposed VRI-2		40.5 (15/37)	1 year
RPL-2		0.0 (0/21)	1 year
Control RIR	1951	7.3 (3/41)	1 year
Exposed RIR		0.0 (0/46)	1 year
Exposed VRI-3 **		10.0 (4/40)	1 year
BB-E1		8.6 (2/23)	1 year
RTF-2	1951	1.0 (1/100)	15 months
BB-W1		36.8 (14/38)	13 months

* Numerator of fraction refers to the number of tumor cases determined at autopsy while the denominator indicates the total number of birds observed.

** The control VRI-2 birds above also served as isolated controls for this group.

The results of hatching RIR and VRI eggs with BB-E eggs and then raising these birds together do not indicate that any definite increase in lymphomatosis occurred (table 1). The very low loss due to this disease in the BB-E1 birds may suggest that they were not infected or that they were more resistant than the BB-W1 birds (table 1). Since the VRI stock was known to be quite susceptible it would appear that the agent was not present.

The effect of selecting eggs from a flock with a low prevalence of lymphomatosis and hatching and raising the offspring in strict isolation is indicated by a 1 per cent occurrence of this disease (RTF-2 birds, table 1). At the present time, this method of selection and isolation appears to be one of the better means of controlling lymphomatosis.

However, unless the parent stock exhibits a low level of lymphomatosis, there is still the possibility of this disease appearing in the offspring, although very satisfactory means of isolation are carried out. The BB-W1 birds (table 1) demonstrated this when 36.8 per cent died of lymphomatosis.

It is interesting that the BB-W1 birds which came from a group of hens without RNA in their serums should have so much more lymphomatosis than the BB-E1 birds from hens with RNA in their serums. Since unrelated males were used for siring these two groups of birds, the difference in susceptibility may be related to the genetic pattern. However, since both males were selected from flocks with a history of 30 to 40 per cent lymphomatosis, the possibility of a relationship between susceptibility to lymphomatosis and the apparent inability to form RNA should also be considered.

The data in table 2 suggests that an agent capable of stimulating RNA was transmitted from the RPL-1 eggs to the VRI-1 birds either at the time of hatching or by contact, or both, with the RPL-1 birds. On the basis of the RNT carried out with serums from the Rous-vaccine test birds, it is doubtful that RNA were due to the inoculation of the vaccine. Certainly the VRI-1

** These birds had no demonstrable levels of RNA in their serums during the six-month observation period before eggs were collected for hatching. The male which had no RNA was obtained from the RPL-1 flock with a history of 41 per cent lymphomatosis.

† Selected from the same flock as the VRI-1 eggs.

controls, which were hatched and raised with the RPL-1 birds and then separated and placed in isolation at the time that the RPL-1 birds were vaccinated, must have obtained the agent from this population. Even the inoculation of live Rous virus does not stimulate the formation of RNA in such a high percentage of those inoculated (unpublished data). Also the lack of RNA in VRI-C, sibs of VRI-1 birds, hatched and raised in isolation is further evidence that an agent appears to have been present in the RPL-1 eggs and birds which was capable of stimulating the formation of RNA. The occurrence of RNA in 91 per cent of the VRI-1 birds (table 2) exposed to RPL-1 birds is much greater than the 10 per cent * level of such antibodies in the VRI control laying flock (Kenzy and Neuzil [9]).

The presence of RNA in the RPL-1 birds as much as eighteen months after hatching suggests that an agent was transferred from the egg. The possibility of RNA transmission through the yolk should be considered; however, Andrewes [1] considered such antibodies as being present for only a few weeks in the serum of the young chicks.

TABLE 2—The Occurrence of Rous-Neutralizing Antibodies in Serums from RPL-1, VRI-1, and VRI-C Chickens

Treatment	RPL-1		VRI-1		VRI-C	
	Rous (+)* (No.)	Rous (—)** (No.)	Rous (+) (No.)	Rous (—) (No.)	Rous (+) (No.)	Rous (—) (No.)
Vaccinated	17	21	—	—	—	—
Not vaccinated	15	19	30	3	—	—
Controls	6	8	15	1	0	70
Totals	38	48	45	4	0	70

* Birds with neutralizing antibodies for the Rous virus in their serums.

** Birds with no demonstrable neutralizing antibodies for the Rous virus in their serums.

SUMMARY

1) The loss due to lymphomatosis increased from 3.8 to 29.3 per cent in a population (VRI-1) hatched and raised with birds (RPL-1) from a population with 41 per cent lymphomatosis.

2) Rous-neutralizing antibodies (RNA) were present in 91 per cent of the serums of birds (VRI-1) hatched and raised with a population (RPL-1) in which a 45 per cent level of RNA was demonstrated, while no RNA were demonstrated in the control birds (VRI-C).

3) Lymphomatosis was observed in 40.5 per cent of a population (VRI-2) hatched and raised with birds (RPL-2) from a population (RPL-1) with a 14.4 per cent prevalence of this disease.

4) When eggs, selected from a flock (RTF-1) with a relatively low incidence of lymphomatosis (8 per cent), were hatched and raised in strict isolation, only 1 per cent of the chickens developed this disease within fifteen months.

5) When eggs, selected from a flock (BB-W) with a history of 37 per cent lymphomatosis, were hatched separately and the chickens raised in strict isolation, 36.8 per cent died of this disease in thirteen months.

* Fifteen of 153 serums tested from 1948, 1949, and 1950 VRI populations were found to contain RNA. One of 15 hens which served as a source of VRI-1 and VRI-C birds was found to have RNA in the serum.

References

[1] Andrewes, C. H.: The Occurrence of Neutralizing Antibodies for Rous Sarcoma Virus in the Sera of Young "Normal Chicks." J. Path. and Bact., *48*, (1939): 225-227.

[2] Cole, R. K., and Hutt, F. B.: Evidence That Eggs Do Not Transmit Leukosis. Poult. Sci., *30*, (1951): 205-221.

[3] Cottral, G. E.: Avian Lymphomatosis, Another Egg-Borne Disease. Proc. 53rd Ann. Meet., U. S. Livestock San. A., (1949): 183-192 (see also Ann. New York Acad. of Sci., *55*, (1952): 221-234.).

[4] Doyle, L. P.: Neuritis or Paralysis of Fowls. Poult. Sci., *8*, (1929): 159-160.

[5] Duran-Reynals, F.: Neutralization of Tumor Viruses by the Blood of Normal Fowls of Different Ages. Yale J. Biol. and Med., *13*, (1940): 61-76.

[6] Duran-Reynals, F.: Yale University School of Medicine, New Haven, Conn. 1949. Information concerning the technique of the Rous-serum neutralization test. Private communication.

[7] Feldman, W. H., and Olson, C., Jr.: Neoplastic Diseases of the Chicken. *In* Biester, H. E., and Schwarte, L. H.: Diseases of Poultry. 2nd ed. The Iowa State College Press, Ames, Iowa, (1948): 637-714.

[8] Kenzy, S. G.: Studies in Avian Neoplasia. I. A Quantitative Evaluation of Neutralizing Antibodies for the Rous-Sarcoma Virus in Avian Serums. Am. J. Vet. Res., *13*, (1952): 388-394.

[9] Kenzy S. G., and Neuzil, P. V.: Studies in Avian Neoplasia. II. Incidence of Rous-Virus Neutralizing Antibodies in Serums Collected from Flocks Experiencing Losses Due to Lymphomatosis. Am. J. Vet. Res., *14*, (1953): 123-128.

[10] Maginson, G. C., and McGaughey, C. A.: Field Observations on the Spread of Fowl Paralysis by Eggs and Young Chicks. Vet. Rec., N.S. *11*, (1931): 573-574.

[11] Warrack, G. H., and Dalling, T.: So-called "Fowl Paralysis," Also Called Neuritis in Chickens, Range Paralysis, Neuro-Lymphomatosis Gallinarum. Vet. J., *88*, (1932): 28-43.

[12] Waters, N. F., and Prickett, C. O.: The Development of Families of Chickens Free of Lymphomatosis. Poult. Sci., *23*, (1944): 321-333.

[13] Waters, N. F.: Natural Transmission of Avian Lymphomatosis. Poult. Sci., *24*, (1945): 226-233.

Antigenic Studies Using Ammonium Sulfate. I. The Relative Sedimentation Effect of Ammonium Sulfate on the Various Antigenic Types of *Salmonella Pullorum*

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ANTIGENIC variants are of particular importance when they exist in the causative organism of any disease, the detection or control of which is based on the use of serological methods. The recognition of serological differences among strains of *Salmonella pullorum* has stimulated additional interest in the field of antigenic variation.

Pullorum disease, of which *S. pullorum* is the causative organism, was first described by Rettger [1] a half century ago and remains among the most costly diseases affecting poultry, the cost depending not only upon direct loss of birds, but also upon the expense of control measures. In recent years, considerable progress has been made in the control and thus toward eventual eradication of this disease through the use of a blood-testing program employing the agglutination test. Infected birds are detected by this test and then eliminated from the breeding stock which otherwise serves as a reservoir for the infection.

Frequently, bacteria which are otherwise identical and are classified in the same species exhibit a distinct antigenic difference, and this difference, as pointed out by Landsteiner,[2] may be the sole criterion for differentiation of types. Less frequently, antigenic variation in the members of an individual species may be associated with differences in cultural and pathogenic behavior.

Well-established procedures for distinguishing the antigenic structure of closely related or culturally identical species of bacteria involve the use of serological techniques. This is exemplified by the presently employed Kauffmann-White Schema for characterizing the antigenic nature of the numerous members of the Salmonella group, many members of which are quite closely related antigenically.

Form variation, a quantitative variation in a somatic antigenic component, is quite frequently noted among member of the Salmonella group of organisms. Kauffmann [3] reported in 1941 that form variation may be encountered in Salmonella strains possessing the somatic antigenic component XII of the Kauffmann-White Schema. With reference to that component, Kauffmann pointed out that the form variation does not involve the entire component but rather a partial antigen which he designated XII_2. The remaining two partial antigens of the component XII, which the author called XII_1 and XII_3, were not found subject to variation.

Among the Salmonella strains possessing only partial antigens XII_1 and XII_3, no variation was noted; however, there was a form variation in various types possessing XII_1, XII_2 or XII_1, XII_2, XII_3. Using absorbed serums, Kauffmann studied the XII partial antigens of a large number of Salmonella strains. However, an analysis of the partial XII antigens of *S. pullorum* was not included in this report.

Younie,[4, 5] a Canadian investigator, first presented definite evidence that there exists an antigenic variation among *S. pullorum* strains and was able to demonstrate that birds infected with the antigenic variant might not be detected when tested with antigen prepared from the usual or standard antigenic form of *S. pullorum*.

The discovery of serological variation in *S. pullorum* cultures prompted extensive studies by other Canadian investigators including Gwatkin,[6–11] Gwatkin and Bond,[12, 13] Gwatkin and Younie,[14] Wright,[15–19] Wright *et al.*,[20] and Byrne.[21] The variant type of pullorum disease was later recognized in the United States.

All of the experimental work both in this country and Canada has confirmed the earlier work of Younie [4, 5] and emphasized the following important facts: (1) There exists a definite antigenic variation among *S. pullorum* strains which can be readily demonstrated serologically; (2) the antigenic variants do not differ from the usual strains in cultural, biochemical, pathogenic, or other characteristics; and (3) an antigen containing the variant type of the pullorum organism should be employed to detect variant infection where it is known to exist in a flock in order to insure the efficiency of the control program for the disease.

Edwards and Bruner [22] in 1946 demonstrated that the complete antigenic formula of *S. pullorum* is IX, XII_1, XII_2, XII_3. Partial antigen XII_2 was found to be subject to variation, and strains possessing a large amount of antigen XII_2 were designated variant strains, while those possessing a well-developed XII_2 antigen were designated standard strains.

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It was further noted that, as a rule, strains possessing a large amount of the XII_2 antigen possessed little XII_3 factor, and that XII_3 strains possessed little XII_2 factor. No variation was noted in the components IX and XII_1. Since most of the agglutinin production during natural infection seems to be stimulated by the partial antigens XII_2 and XII_3, the importance of this variation in pullorum disease testing is evident.

The serological differentiation of the antigenic forms of *S. pullorum* employing the methods of Edwards and Bruner,[22] later detailed by Wright and Edwards,[23] involves the use of two antiserums, one containing XII_2 agglutinins and another containing XII_3 agglutinins. The rapid macroscopic plate technique, or, less frequently, the tube agglutination test is employed.

From the foregoing discussion and as illustrated in more detail by the results of experimental studies to be reported in this paper, it is evident that three basic antigenic forms of *S. pullorum* exist. Standard strains, subsequently designated (S), are composed almost entirely of cells having XII_3 antigenic characteristics. This is the most frequently encountered antigenic form of the organism. Variant strains, subsequently designated (V), are predominantly composed of cells having XII_2 characteristics.

Intermediate *S. pullorum* strains, subsequently designated (I), contain both XII_2 and XII_3 cells either balanced in almost equal proportions or the two types of cells may be present in widely varying proportions. The XII_2 and XII_3 type cells may be found in the same single colony or each in separate individual colonies when the cultures are plated and individual colonies typed.

The I antigenic form of *S. pullorum* represents a stage of active antigenic variation of the organism, and I strains will often be noted to change in their balance of XII_2 and XII_3 cells following several serial transfers and are capable of readily giving rise to both S and V forms.

I antigenic forms of *S. pullorum* are probably encountered more frequently than is indicated in the literature. The recent work reported by Snoeyenbos *et al.*[24] indicates the wide variation of XII_2 and XII_3 cells that may exist in freshly isolated cultures of *S. pullorum*. These investigators were able to demonstrate a variation in the antigenic balance of strains of *S. pullorum* isolated from different organs of the same infected bird, and also noted a variation in strains of the organism periodically isolated from the eggs of the same infected hens.

I strains may at times be designated V forms, especially when single colony typing is not routinely done or only one single factor serum is used in typing. This practice is perhaps partially justified by the fact that I antigenic forms are potentially capable of giving rise to V type strains, in contrast to S and V cultures which are, in general, regarded as existing in a more stable state. Snoeyenbos *et al.*[25] chose to include in a group which they called nonvariants all cultures considered to be standard and also those undergoing active form variation.

Prior to this time, differentiation of the different antigenic types of *S. pullorum* has been accomplished solely by serological methods. In the course of work recently carried out, differences of a previously undescribed nature have been recognized. It is the purpose of this paper to describe and discuss these differences.

During the progress of recent studies of the relative value of various chemical agents in increasing the sensitivity and improving the keeping quality of pullorum whole blood antigens for turkey testing, ammonium sulfate* was one of the chemical agents under investigation. S, V, and I antigenic forms of *S. pullorum* were being used in this work.

The observation was made that when S type suspensions of *S. pullorum* composed predominantly of XII_3 type cells were exposed to aqueous solutions of ammonium sulfate containing approximately 300 Gm. of the salt per liter, there occurred a marked flocculation and sedimentation of the cells leaving after a short period of centrifugation a clear supernatant fluid.

Strains of *S. pullorum* serologically classified as V types and composed predominantly of XII_2 type cells revealed little or no flocculation or sedimentation when exposed to the above mentioned concentration of ammonium sulfate, and consequently the supernatant fluid remained markedly turbid. Extended centrifugation of the V type cells in the ammonium sulfate solution did not clear the supernatant

* The polyvalent salt, ammonium sulfate ($[NH_4]_2SO_4$), has for a number of years been employed in the fractionation of proteins by its selective "salting out" action.

fluid of the XII_2 organisms although some did settle. Identical suspensions in distilled water were readily cleared by centrifugation.

When I type cultures were tested there was, as a rule, a definite flocculation of less degree than noted with S type cultures, and a definitely turbid supernatant fluid remained following centrifugation. However, the turbidity of the supernatant fluid was not as marked as that noted with V type suspensions.

These preliminary observations indicated that strains of *S. pullorum* classified as S serologically are more susceptible to the sedimenting effect of ammonium sulfate than are V or I type cultures. The sensitivity to ammonium sulfate of I type cultures is characteristic to a varying degree of both S and V strains, confirming the fact that such cultures are composed of a mixture of XII_2 and XII_3 type cells.

EXPERIMENTAL PROCEDURE

Medium.—All cultures of *S. pullorum* used in these studies were grown on the K type medium** first described by MacDonald.[26] K medium will support luxuriant growth of most *S. pullorum* strains and was used for this reason. Some strains of *S. pullorum* will not grow well on this medium.

When a large quantity of the organisms was desired, the medium was dispensed in 100-cc. portions into 300-cc. Erlenmeyer flasks and each flask seeded with a separate *S. pullorum* strain. In preparing seed cultures, tubes of beef infusion broth were inoculated from fresh agar slant cultures. After a 24-hour incubation period at 37 C. the broth cultures were used to inoculate the flasks of K medium using approximately 0.5 cc. of broth for each flask, and flowing it over the surface as uniformly as possible. The flasks were capped with foil and incubated at 37 C. for forty-eight hours.

Harvesting the Growth.—In harvesting the growth, 25 cc. of sterile phosphate buffer solution (pH 6.4) having the following composition was added to each flask:

NaCl		7.5 Gm.
KH_2PO_4		1.9 Gm.
Na_2HPO_4		1.1 Gm.
Formalin†		10.0 cc.
Distilled H_2O	q. s. ad	1000.0 cc.

It was not found necessary to either filter or wash the bacterial suspensions prepared in this way for use in these studies. Hydrogen ion determinations on the bacterial suspensions immediately following harvest revealed an average pH reading of 5.8.

Standardization of Suspensions.—The bacterial suspensions were carefully standardized by visual methods to 100 x tube No. 1 of the McFarland scale,[27] using the buffer solution as the diluent. The suspensions so standardized were subsequently checked using the Klett-Summerson colorimeter with a satisfactory degree of agreement between the two methods.

With reference to the preparation of the McFarland standard, it should be pointed out that in practice a suspension of killed *S. pullorum* organisms is prepared so that it matches the chemical No. 1 standard of the McFarland scale and this bacterial substandard is used in routine work. The diluent used is an 0.85 per cent sodium chloride solution containing 1 per cent formalin. The chemical standard is used for standardizing the bacterial substandard within fifteen to thirty minutes after preparation. The bacterial substandard is checked at intervals against a freshly prepared chemical standard and at three-month intervals new bacterial standards are prepared.

Ammonium Sulfate Solutions.—All concentrations of ammonium sulfate solutions used in these studies will be expressed in grams per liter (Gm./l.). The designation 300 Gm./l. indicates that 300 Gm. of analytical grade ammonium sulfate was placed in a 1-liter volumetric flask and distilled water added to give a final volume of 1 liter.

Method of Testing.—The tests were conducted in the following manner. Chemically clean, pyrex serological tubes (100 mm. by 13 mm.), free of excessive scratch marks and etching, were set up in wire racks and labeled. One tube was used for each concentration of ammonium sulfate to be studied.

One-half cc. of the standardized bacterial suspension was placed in each tube and 8 cc. of ammonium sulfate solution of the desired concentration was added. Sufficient mixing usually resulted from the addition of the ammonium sulfate solution.

The tubes were immediately placed in a constant temperature water bath set at 55 C. and allowed to remain for five minutes. After the incubation period, the tubes were removed from the water bath and immediately observed for flocculation against a bright background. The tubes were not agitated in making the readings. The degrees of flocculation noted in the tubes were recorded as ±, +, and ++, indicating a trace, a moderate, and a marked degree of flocculation, respectively. If no flocculation was noted, a negative reading was recorded.

Following the period of initial observation, the tubes were immediately centrifuged at 2,400 r.p.m. (size 2 International centrifuge) for fifteen minutes. Final readings of the degrees of turbidity of the supernatant fluids were made against a

** The method of preparing this medium may be secured from the Pathological Division, Bureau of Animal Industry, U.S.D.A., Washington 25, D. C.

† Containing approximately 40 per cent formaldehyde with pH adjusted to about 6.5 with Na OH.

bright background when the tubes were removed from the centrifuge. The turbidity of the supernatant fluid was recorded as ±, +, or ++ depending upon the degree of turbidity noted. If no turbidity was noted, a negative reading was recorded. With a little experience, one is able to make observations regarding the degrees of flocculation and turbidity which for all practical purposes are sufficiently accurate.

Selection of S, V, and I Cultures.—In selecting S, V, and I antigenic forms of *S. pullorum* for these studies, single colony serological typing using XII_2 and XII_3 single factor serums was employed. Fifty single colonies were selected from each strain for typing using essentially the technique described by Snoeyenbos *et al.*[24]

Twelve S strains, 12 V strains, and eight I strains were serologically studied and selected for testing. The results of single colony examination of these strains will not be detailed here; however, some of the pertinent information derived from this work should be included in this report.

Of the 12 S type cultures examined, only four revealed the presence of XII_2 type cells detectable by the serological techniques used. In no case did the number of XII_2 or XII_2-XII_3 type colonies exceed seven out of 50 in any of the S type cultures examined.

The single colony serological examination of the V strains revealed that eight of the 12 cultures contained some XII_3 cells with, however, a marked dominance of XII_2 type cells. Both XII_2 and XII_3 type cells were found to be present in single colonies and, furthermore, XII_2 and XII_3 single factor colonies were encountered in the same variant strain. The number of colonies revealing XII_3 type cells was in no case found to exceed ten of the 50 examined.

The foregoing findings are in accord with the recent typing results of Sudds and Jungherr,[28] who were able to demonstrate some XII_3 factor in the cultures which they typed and found to be predominantly in the XII_2 form. No XII_2 factor was demonstrated in cultures typed as XII_3 or standard form.

In the selected I strains, XII_2 type cells were, in most cases, found to slightly predominate. In only one of the cultures was the number of XII_3 colonies slightly greater than XII_2 colonies. Four of the eight I suspensions were prepared by mixing equal volumes of standardized suspensions of S and V cultures.

TABLE 1—The Relative Sedimentation Effect of Varying Concentrations of Ammonium Sulfate on Standard, Variant, and Intermediate Antigenic Types of Salmonella Pullorum

	S Type		V Type		I Type	
$(NH_4)_2SO_4$ Gm./l.	Degree flocculation*	Supernat. turbidity**	Degree flocculation	Supernat. turbidity	Degree flocculation	Supernat. turbidity
250	±	++	−	++	±	++
260	±	++	−	++	±	++
270	+	+	−	++	+	+
280	++	±	−	++	+	+
290	++	±	−	++	+	+
300	++	±	−	++	+	+
310	++	−	−	++	+	+
320	++	−	−	++	+	+
330	++	−	−	++	+	+
340	++	−	−	++	+	+
350	++	−	−	++	+	+
360	++	−	−	++	+	+
370	++	−	−	++	+	+
380	++	−	−	++	+	+
390	++	−	−	++	+	+
400	++	−	±	++	+	+
410	++	−	±	++	+	+
420	++	−	±	++	+	+
430	++	−	±	++	+	+
440	++	−	±	+	+	±
450	++	−	+	+	++	±
460	++	−	+	±	++	−
470	++	−	++	−	++	−
480	++	−	++	−	++	−
490	++	−	++	−	++	−
500	++	−	++	−	++	−
To saturation	++	−	++	−	++	−

* − = No flocculation.
± = Trace of flocculation.
+ = Moderate flocculation.
++ = Marked flocculation.

** − = Clear supernatant.
± = Trace of turbidity.
+ = Moderate turbidity.
++ = Marked turbidity.

The foregoing results further confirm the wide variation of antigenic balance that may be noted among *S. pullorum* strains. The S strains studied were closer to being "pure" XII_3 types than were the V strains to being "pure" XII_2 types, since the V strains more frequently contained XII_3 type cells.

EXPERIMENTAL RESULTS

The relative sedimenting effect of varying concentrations of ammonium sulfate on typical S, V, and I antigenic types of *S. pullorum* is indicated in table 1. In the lower concentrations of ammonium sulfate, that is, 250 Gm./l. to 300 Gm./l., S and I types of *S. pullorum*, by virtue of their XII_3 cell content, reacted in a very similar manner.

At a concentration of approximately 310 Gm./l., S strains usually exhibited a marked flocculation after a five-minute period of incubation at 55 C. and a clear supernatant fluid after a fifteen-minute period of centrifugation at 2,400 r.p.m. I strains were not cleared by this concentration of ammonium sulfate and a moderate supernatant turbidity remained after centrifugation.

Some S type cultures were encountered which were more sensitive to ammonium sulfate sedimentation than indicated above. These strains were found to be cleared by concentrations slightly below 310 Gm./l. but in no case below 280 Gm./l. No culture serologically typed as S which was not cleared by 330 Gm./l. ammonium sulfate was encountered. This concentration of ammonium sulfate has, therefore, been selected as the diagnostic concentration for use in typing *S. pullorum* cultures to be discussed in a second paper of this series.

V type *S. pullorum* cultures, in contrast to S type, revealed very little or no flocculation in ammonium sulfate concentrations below 400 Gm./l. following incubation for five minutes at 55 C. A concentration of ammonium sulfate approximating 470 Gm./l. was necessary to clear the supernates of V suspensions following incubation and centrifugation.

Some fine granulation with clear interstitial fluid was occasionally noted in the supernatant fluid of V strains following centrifugation in 470 Gm./l. ammonium sulfate. Below the concentration of 470 Gm./l. ammonium sulfate, a definite turbidity was noted in the supernatant fluids of variant strains. As the concentration of 470 Gm./l. was approached, this turbidity became less marked.

The results obtained on testing I type strains during this study indicate, as does serological typing, the marked variability of the antigenic balance of such cultures. The data presented in table 1 are typical of an I strain in which XII_2 and XII_3 type cells were well balanced. Many of the I type cultures encountered revealed a dominance of V type cells as judged by the turbidity of the supernatant fluids following centrifugation in 330 Gm./l. ammonium sulfate.

Intermediate type cultures with a dominance of XII_3 type cells will generally reveal a marked flocculation in 330 Gm./l. ammonium sulfate and a slight degree of turbidity in the supernatant fluid following centrifugation. Such cultures have not been frequently encountered but were readily produced by mixing S and V standardized suspensions.

It is evident from the foregoing discussion that an ammonium sulfate concentration of 310 Gm./l. will clear most suspensions of S type *S. pullorum* cultures, have little effect on the turbidity of V cell suspensions, and only partially clears I type cultures. An ammonium sulfate concentration of 470 Gm./l. will completely clear S, V, and I type culture suspensions.

Gram staining of the sediment of S strains and the turbid supernatant fluids of V strains revealed the presence of gram-negative bacilli showing no distinguishing differences in morphology or other characteristics. It appears, therefore, that the exposure to ammonium sulfate did not change the gram-staining characteristics of the organism. Microscopic examination of S type cells suspended in 330 Gm./l. ammonium sulfate using dark field technique permits one to observe the cells forming into aggregates. V type cells treated in the same way are observed to remain separated in the suspensions.

Concentrations of ammonium sulfate below 250 Gm./l. will have little effect on most strains of *S. pullorum*, irrespective of their antigenic characteristics. Some S and I antigenic types may reveal traces of flocculation.

DISCUSSION

The data presented in table 1 are typical of the results obtained with most S, V, and I antigenic types of *S. pullorum* when tested by the methods outlined. However, it should be pointed out that a few S strains were encountered which revealed only a moderate degree of flocculation after the short incubation period of only five minutes in 330 Gm./l. ammonium sulfate. These same strains were consistently cleared by more prolonged incubation or by centrifugation for fifteen minutes.

It would appear, therefore, that the degree of turbidity of the supernatant fluids following incubation and centrifugation should be regarded as a more consistent and reliable characteristic than flocculation for distinguishing S, V, and I antigenic forms of *S. pullorum* by the experimental methods outlined here.

Since all V and I type cultures maintained turbid supernates following centrifugation in 330 Gm./l. ammonium sulfate in contrast to cleared S strains, the degree of turbidity was typical of their relative XII_2 cell content. This was readily demonstrated in the case of I type cultures by testing mixed suspensions composed of S and V type cells added in varying proportions.

The experimental work reported herein substantiates and adds to data established by other workers which indicate the distinct antigenic differences that characterize S, V, and I types of *S. pullorum*. The new techniques presented have proved useful and reliable in classifying the various antigenic types of *S. pullorum* and do not involve the use of conventional serological methods. Consequently, evidence is presented of a previously undescribed fundamental difference that exists in the various antigenic forms of the organism.

Concurrent with antigenic variation of *S. pullorum* cultures, there occurs a change in the susceptibility of the cultures to the sedimenting effect of certain concentrations of the salt ammonium sulfate. An explanation of this phenomenon should provide additional insight into the basic physical or chemical changes that occur in bacterial cells during antigenic variation, and probably aid in the explanation of those factors which promote such variation. The mechanism involved is not clearly understood at this time.

The experimental procedures described in this paper may possibly be employed to characterize other closely related organisms, besides those of the Salmonella group, in which form variation and minor antigenic differences exist. No doubt other salts and chemical agents will provide results similar to those obtained in these studies, and should be investigated. Techniques of more quantitative value than those presented herein may also be developed.

SUMMARY

A previously undescribed fundamental difference of a nonserological nature which was found to exist in the various antigenic types of *S. pullorum* is discussed in this paper. The relative susceptibility of the different antigenic forms of the organism to the flocculating and sedimenting effects of certain concentrations of ammonium sulfate was correlated with the relative balance of XII_2 and XII_3 type cells in the cultures.

Suspensions of standard, variant, and intermediate antigenic forms of *S. pullorum* were exposed to varying concentrations of ammonium sulfate, incubated for a short time and then centrifuged. It was found that a concentration of ammonium sulfate approximating 310 Gm./l. completely cleared the supernatant fluids of standard type suspensions, had little or no effect on variant suspensions, and only partially cleared intermediate suspensions. A concentration of the salt approximating 470 Gm./l. was required to clear suspensions of variant and intermediate type cultures.

References

[1] Rettger, L. F.: Septicemia Among Young Chickens. New York Med. J., *71*, (1900): 803-805.

[2] Landsteiner, Karl: The Specificity of Serological Reactions. Revised ed. 1947. Harvard University Press, Cambridge, Mass.

[3] Kauffmann, F.: A Typhoid Variant and a New Serological Variation in the Salmonella Group. J. Bact., *41*, (1941): 127-140.

[4] Younie, A. R.: Fowl Infection Like Pullorum Disease. Canad. J. Comp. Med., *5*, (1941): 164-167.

[5] Younie, A. R.: Pullorum Like Infection of Fowl. Canad. J. Comp. Med., *6*, (1942): 172-173.

[6] Gwatkin, R.: Studies in Pullorum Disease. V. Efficiency of Homologous and Heterologous Antigens in Detecting Reacting Birds in a Variant Infected Flock. Canad. J. Comp. Med., *9*, (1945): 183-191.

[7] Gwatkin, R.: Studies in Pullorum Disease. VI. Comparison of Whole Blood and Tube Tests with Regular and Variant Antigen and a Combination of the Two Antigens. Canad. J. Comp. Med., *9*, (1945): 216-223.

[8] Gwatkin, R.: Studies in Pullorum Diseases. IX. Serological Reactions of Regular and Variant Types of *Salmonella Pullorum*. Canad. J. Comp. Med., *10*, (1946): 254-267.

[9] Gwatkin, R.: Studies in Pullorum Disease. X. Bacteriological Examination of Tissues from Fowls Exposed to Infection with *Salmonella Pullorum*. Canad. J. Comp. Med., *10*, (1946): 277-283.

[10] Gwatkin, R.: Resumé of Studies Relating to Younie Strains of *Salmonella Pullorum*. Proc., 18th Ann. Meet. Northeast Conf. of Lab. Workers in Pullorum Disease Control, 1946.

[11] Gwatkin, R.: Studies in Pullorum Disease. XII. Antigenic Differences in Strains of *Salmonella Pullorum*, Am. J. Vet. Res., *8*, (1947): 204-208.

[12] Gwatkin, R., and Bond, E. W.: Studies in Pullorum Disease. III. Serological Differences in Strains of *Salmonella Pullorum*. Canad. J. Pub. Health, *36*, (1945): 160-166.

[13] Gwatkin, R., and Bond, E. W.: Studies in Pullorum Disease. XIX. Examination of Colonies from Regular and Variant Form Subcultures of *Salmonella Pullorum*. Canad. J. Comp. Med., *11*, (1947): 282-289.

[14] Gwatkin, R., and Younie, A. R.: Studies in Pullorum Disease. XVII. The Use of *Salmonella Typhi* 090I in Test Antigens. Canad. J. Comp. Med., *11*, (1947): 172-178.

[15] Wright, M. L.: Pullorum-like Variants and Nonspecific Reactors. Proc., 16th Ann. Meet. Northeast Conf. of Lab. Workers in Pullorum Disease Control. 1944.

[16] Wright, M. L.: Selection of Suitable Antigenic Strains of *S. Pullorum* by Single Colony Isolation. Canad. J. Comp. Med., *11*, (1947): 68-74.

[17] Wright, M. L.: The Suitability of *Salmonella Pullorum* Strains Used in the Agglutination Test for Pullorum Disease Control. J.A.V.M.A., *112*, (1948): 241-244.

[18] Wright, M. L.: Testing for Standard and Variant *S. Pullorum* Infection with a Single Tube Antigen. Proc., 21st Ann. Meet. Northeast Conf. of Lab. Workers in Pullorum Disease Control. 1949.

[19] Wright, M. L.: Serological Examination of *S. Pullorum* Strains Received from Various States of the U. S. A. Proc., 17th Ann. Meet. Northeast Conf. of Lab. Workers in Pullorum Disease Control. 1945.

[20] Wright, M. L., Garrard, E. H., and Marcellus, F. N.: Preliminary Report on a Pullorum-like Organism Causing Infection in Fowl. Proc., 15th Ann. Meet. Northeast Conf. of Lab. Workers in Pullorum Disease Control. 1942.

[21] Byrne, J. L.: Variant or Atypical Strains of *Salmonella Pullorum*. A Serological Study. Canad. J. Comp. Med., 7, (1943): 227-238.

[22] Edwards, P. R., and Bruner, D. W.: Form Variation in *Salmonella Pullorum* and Its Relation to X Strains. Cornell Vet., *36*, (1946): 318-324.

[23] Wright, M. L., and Edwards, P. R.: The Serologic Differentiation of *Salmonella Pullorum* Forms. Am. J. Vet. Res., *9*, (1948): 386-388.

[24] Snoeyenbos, G. H., Crotty, A. M., and Van Roekel, H.: Some Antigenic Characteristics of *Salmonella Pullorum*. Am. J. Vet. Res., *11*, (1950): 221-225.

[25] Snoeyenbos, G. H., Bachman, B., and Van Roekel, H.: A Survey of the Incidence of Antigenic Forms of *Salmonella Pullorum* Occurring in the United States. Proc., 23rd Ann. Meet. Northeast Conf. of Lab. Workers in Pullorum Disease Control. 1951.

[26] MacDonald, A. D.: K Antigen for the Detection of Pullorum Disease in Poultry. Proc. 19th Ann. Meet. Northeast Conf. of Lab. Workers in Pullorum Disease Control. 1947.

[27] McFarland, John: The Nephelometer: An Instrument for Estimating the Numbers of Bacteria in Suspensions Used for Calculating the Opsonic Index and for Vaccines, J. Am. M. A., *49*, (1907): 1176-1178.

[28] Sudds, Dorothy, and Jungherr, E. L.: Typing of *Salmonella Pullorum* and *Salmonella Gallinarum* Cultures Isolated in Connecticut in 1950 and 1951. Proc., 24th Ann. Meet. Northeast Conf. of Lab. Workers in Pullorum Disease Control. 1952.

Antigenic Studies Using Ammonium Sulfate. II. The Macroscopic Ammonium Sulfate Sedimentation Test for Distinguishing the Antigenic Forms of *Salmonella Pullorum*

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THE LITERATURE relating to antigenic variation in cultures of *Salmonella pullorum* has been reviewed in a previous paper [1] of this series. In that publication, it was pointed out that presently employed procedures for differentiating standard (S), variant (V), and intermediate (I) antigenic types of *S. pullorum* involve the use of serological methods. These procedures are based on form variation in somatic antigenic component XII as first described by Kauffmann.[2]

In early studies of antigenic variants of *S. pullorum*, it was established by Younie,[3] Byrne,[4] and others, that the various antigenic forms of the organism possess no unique cultural or physiological characteristics that provide a means of distinguishing one from the other. Neither has it been demonstrated that there is a difference in virulence.

Gwatkin [5] tried unsuccessfully to differentiate S and V strains of *S. pullorum* through the use of bacteriophage. The present author and Jones [6] conducted similar studies testing 280 serologically typed strains of *S. pullorum* against 16 strains of pullorum bacteriophage which had been isolated by single plaque selection. It was not possible to demonstrate any correlation between antigenic structure of the strains tested and their bacteriophage susceptibility.

Gwatkin [7] cultivated S and V forms of *S. pullorum* on homologous and heterologous serum agar; however, such procedures were found to be of no value in differentiating the antigenic forms of the organism. In further experimental work, the same investigator found that no change in the antigenic form of *S. pullorum* cultures resulted from passage of the organisms through mice, guinea pigs, and chickens in the presence of large quantities of killed heterologous form cultures. Twenty serial subcultures on agar and in broth containing killed V culture did not modify the form of an S strain of *S. pullorum*.

Edwards and Bruner [8] demonstrated in 1946 that the partial antigen XII_3 predominates in S strains, while V strains of the organism exist mainly in the XII_2 form, and I strains exist in an active state of variation exhibiting a varying balance of both XII_2 and XII_3 forms. I strains are rather unstable and are capable of giving rise to both S and V form cultures.

The serological differentiation of *S. pullorum* strains involves the use of two antiserums, one containing the XII_2 agglutinins and the other the XII_3 agglutinins. Procedures for this technique are described in the paper by Wright and Edwards.[9] The rapid macroscopic plate agglutination test is generally employed.

Serological typing of the various antigenic forms of *S. pullorum* as now practiced is mainly a qualitative technique. Observations by Edwards *et al.*,[10] Wright and Edwards,[9] and Snoeyenbos *et al.*[11] indicate that one must not depend merely upon a cursory examination of a *S. pullorum* culture in determining its true antigenic balance.

The suggestion was made by Wright and Edwards [9] that at least 100 colonies from a given culture be individually examined serologically in reaching a decision regarding the relative balance of XII_2 and XII_3 antigenic forms in the culture. A technique such as the above is not practical for the average laboratory when a rapid diagnosis regarding the antigenic nature of *S. pullorum* strains is desirable.

Serological typing using the rapid macroscopic plate technique, even when single colony selection is practiced, does not provide an accurate quantitative evaluation of the balance of XII_2 and XII_3 type cells in *S. pullorum* cultures. Snoeyenbos *et al.*[11] observed that "birds infected with *S. pullorum* not of a variant form may yield cultures with almost any ratio between XII_2 and XII_3 antigens; this being a variable factor concise classification of these forms is impracticable."

Single colony typing does provide one with a relative qualitative evaluation of the XII_2-XII_3 antigenic balance in a culture. Consequently, some investigators find single colony typing more useful in reaching a decision regarding the balance of XII_2 and XII_3 cells than composite, that is, direct typing of isolated strains.

Snoeyenbos *et al.*[11] reported the isolation of *S. pullorum* strains which were insensitive to the typing serums. These same authors cite references to emphasize the fact that the terms used to designate the various antigenic forms of *S. pullorum* vary widely. Snoeyenbos *et al.*[11, 12] chose to include in a nonvariant group all cultures considered serologically to be S and also those undergoing active form variation.

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THE MACROSCOPIC AMMONIUM SULFATE SEDIMENTATION TEST

In the previous paper [1] of this series, the results of studies of the relative sedimenting effect of varying concentrations of the salt, ammonium sulfate, on S, V, and I antigenic forms of *S. pullorum* were reported. It was noted that S strains of *S. pullorum* were more rapidly and completely flocculated by lower concentrations of ammonium sulfate than were suspensions of V and I cultures.

The initial observations suggested that techniques employing ammonium sulfate might be adapted for routinely typing the various antigenic forms of *S. pullorum*. Information regarding the antigenic nature of *S. pullorum* strains is often of considerable importance to the poultry pathologist engaged in the laboratory phases of pullorum disease control work.

It is the purpose of this paper to describe and discuss a new method using ammonium sulfate which has been found reliable for typing the various antigenic forms of *S. pullorum*.

Solutions and Equipment.—The solutions and equipment employed in conducting the macroscopic ammonium sulfate sedimentation test are illustrated in figure 1. They are as follows:

1) Sterile Phosphate Buffer Solution (pH 6.4).—Directions for the preparation of the buffer are presented in the previous paper [1] of this series.

2) Ammonium Sulfate Test Solution.—Accurately weigh 330 Gm. of ammonium sulfate, analytical reagent grade, and place it in a 1-liter chemically clean volumetric flask. Add distilled water to dissolve the salt and adjust the volume to 1 liter. Filter the test solution through filter paper and store at room temperature in a tightly stoppered container to prevent evaporation. The solution need not be sterilized. As prepared in this laboratory, the pH value of the solution is approximately 5.3 (Beckman glass electrode meter).

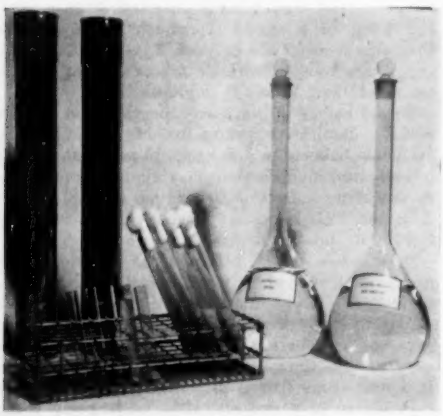

Fig. 1—Solutions and equipment for conducting the macroscopic ammonium sulfate sedimentation test.

3) Graduated Pipettes (1 cc. and 10 cc.).—Chemically clean and sterile.

4) Serological Test Tubes (100 mm. by 13 mm.).—Pyrex, chemically clean, and free of excessive scratch marks and etching.

Preparation of Cell Suspensions for Typing.—Culture Mediums.—The K type medium,* discussed in a previous paper,[1] has been found very satisfactory for growing cultures to be typed. It supports luxuriant growth of most *S. pullorum* cultures.

The medium is dispensed in approximately 7-cc. amounts into test tubes (150 mm. by 16 mm.). The tubes are slanted in such a manner that a rather deep butt and a slant about 10 cm. in length are provided.

Other mediums which were studied and found to be as satisfactory for routine use as K type medium were the TG medium* of MacDonald;[13] K type medium substituting anhydrous cysteine-mono hydrochloride, C. P. (2 Gm./l.) in place of colloidal sulfur; blood agar base medium (Difco) with 1 Gm. of colloidal sulfur suspended in 20 cc. of glycerine added to each liter of medium; and blood agar base medium (Difco) with 2 Gm. of anhydrous cysteine-mono hydrochloride suspended in 20 cc. of glycerin added per liter. The agar content of all of the mediums was adjusted to 3 per cent and the pH value to 7.2 before tubing and sterilization.

Inoculation of Medium.—The medium is inoculated by streaking the surface of the slant uniformly with growth from the original agar slant culture of the *S. pullorum* strain to be typed. The culture used for inoculation must be pure and definitely identified as *S. pullorum* by biochemical tests.

Initial cultures made directly from diseased tissues should not be used for typing by this method, since such cultures frequently contain considerable protein material derived from the tissues which were used for isolation of the organism. Extraneous proteins may interfere with the results. The inoculated slants are incubated for twenty-four hours at 37 C.

Harvesting the Growth.—The growth from each agar slant is harvested in 2 to 3 cc. of the formolized buffer solution. In harvesting strains of *S. pullorum* that grow luxuriantly, and this includes most strains, 3 cc. per tube are used, while those strains that grow sparsely are harvested in 2 cc. or less of buffer. The density of the suspensions

* The method of preparing this medium may be secured from the Pathological Division, Bureau of Animal Industry, U.S.D.A., Washington 25, D. C.

should approximate 100 x tube No. 1 of the McFarland scale,[14] and with a little experience one is able to gauge the amount of buffer to add in order to approximate that value.

The growth is carefully detached from the agar surface using a sterile wire loop, following which the tube is vigorously agitated to provide an homogenous suspension of the organisms. The occasional *S. pullorum* strain which does not grow well on K medium may afford sufficient growth if incubated for an additional twenty-four hours at 37 C. before testing, or the culture may be grown satisfactorily on one of the other recommended mediums.

When accurately standardized suspensions are desired, the McFarland optical method is used and the density is adjusted to 100 x tube No. 1 of the McFarland scale. The procedure for making density adjustments using this method can be found in most textbooks on laboratory methods.

Procedures.—Procedure 1, Use of Unstandardized Bacterial Suspensions.—In a wire rack one serological test tube is set up and properly labeled for each culture to be typed. Using a sterile 1-cc. graduated pipette, 0.5 cc. of the bacterial suspension is deposited in each tube. Eight cubic centimeters of 330-Gm./l. ammonium sulfate test solution are added to each tube. Addition of the test solution usually affords sufficient mixing. The pH value of the test mixtures approximates that of the test solution, namely 5.3.

The test tubes are next incubated in a constant temperature water bath at 55 C. for five minutes. Immediately following incubation, they are centrifuged at 2,400 r.p.m. (size 2 International centrifuge) for fifteen minutes. Following removal of the tubes from the centrifuge, the results are read against a bright background. Readings are made as follows (fig. 2):

S Culture.—Characterized by a clear supernatant fluid with sedimented cells packed in the bottom of the tube. A faint trace of turbidity may be noted in the supernatant fluid of some S type cultures.

V or I Cultures.—Characterized by a marked or moderate turbidity of the supernatant fluid with some sedimented cells packed in the bottom of the tube. V type cultures usually reveal very markedly turbid supernatant fluids in contrast to the moderately turbid supernatant fluids of I type cultures.

Procedure 1 affords a method for rapidly separating *S. pullorum* cultures into the two groups described above. It is evident from the foregoing discussion that the degree of turbidity of the supernatant fluid following centrifugation is directly dependent upon the XII_3 cell content of the culture. Since most S strains of *S. pullorum* contain few XII_3 type cells, suspensions of such cultures are rapidly flocculated by the test solution and are cleared by centrifugation.

When unstandardized suspensions are used for testing, the degree of flocculation noted following incubation is not always as reliable a criterion for determining the XII_2-XII_3 cell balance of a cul-

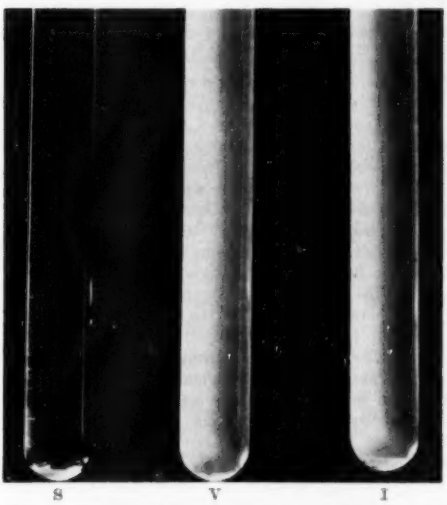

Fig. 2—Macroscopic ammonium sulfate sedimentation tests immediately following centrifugation—(S) standard strain, (V) variant strain, and (I) well-balanced intermediate strain.

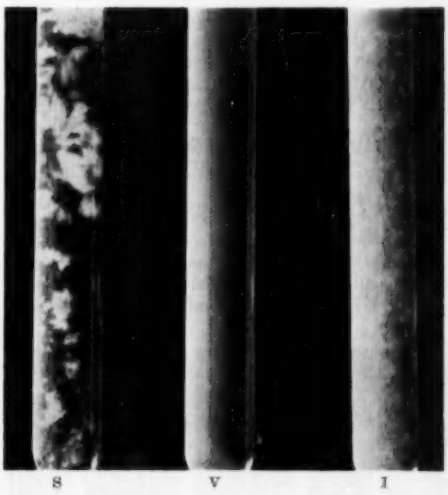

Fig. 3—Macroscopic ammonium sulfate sedimentation tests immediately following incubation and prior to centrifugation—(S) standard strain, (V) variant strain, and (I) intermediate strain.

ture as is the degree of supernatant turbidity noted following centrifugation. However, the degree of flocculation noted with unstandardized suspensions is of value in many cases and may be given consideration.

If the five-minute incubation period is eliminated, the final typing results will be comparable to those obtained when incubation is practiced; however, in final analysis, tests that have been incubated before centrifugation will usually be found more satisfactory.

Procedure 2, Use of Standardized Bacterial Suspensions.—In this procedure, the bacterial suspensions are carefully standardized to a density of 100 x tube No. 1 of the McFarland scale before testing. The procedure for conducting the test is essentially the same as that used in testing unstandardized suspensions. The use of standardized suspensions permits one to more accurately evaluate the XII_2-XII_3 cell balance of an *S. pullorum* culture based on both the degree of flocculation noted following incubation and on the degree of supernatant turbidity following centrifugation.

Immediately following the five-minute incubation period at 55 C., the tubes are observed without agitation against a bright background. As illustrated in figure 3, S type suspensions will, in most cases, reveal a marked flocculation; V type suspensions will be free of flocculation or reveal only a trace; I type suspensions will reveal varying degrees of flocculation depending upon the XII_3 cell content of the cultures.

Following centrifugation, the degrees of turbidity are noted. The supernatant fluids of S type strains will be clear or reveal only a trace of turbidity; those of V type strains will be markedly turbid; those of I type strains will reveal varying degrees of turbidity depending upon the XII_2 cell content of the culture being typed. The varying turbidities noted with various I type suspensions are illustrated in figure 4.

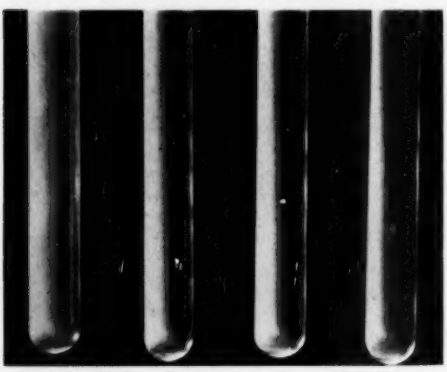

Fig. 4—Completed tests using standardized intermediate type suspensions of Salmonella Pullorum—(1) a mixture of 1 part S cells and 9 parts V cells; (2) a mixture of 4 parts S cells and 6 parts V cells; (3) a mixture of 6 parts S cells and 4 parts V cells; (4) a mixture of 9 parts S cells and 1 part V cells.

In evaluating the relative XII_2 cell balance of I strains, it may be desirable to conduct control tests using mixtures containing varying amounts of standardized suspensions of S and V strains. This will aid one in becoming familiar with the characteristics of various types of I strains that are encountered. S and V type controls may also be desirable to acquaint one with the characteristics of such strains.

Presented in table 1 is a chart listing the characteristics of the various antigenic forms of *S. pullorum*.

Procedure 3.—In this procedure, the tests are conducted as outlined under procedure 1, using either standardized or unstandardized bacterial suspensions. The tests are incubated at room temperature rather than in the water bath and the incubation extended for one hour or even overnight before centrifugation for fifteen minutes at 2,400 r.p.m. After overnight incubation at room temperature, it is usually possible to make final readings of the tests without centrifugation. This procedure permits the separation of *S. pullorum* cultures into the two groups described in procedure 1.

TABLE 1—The Antigenic Analysis of Standard, Variant, and Intermediate Salmonella Pullorum Suspensions (Standardized) Employing the Macroscopic Ammonium Sulfate Sedimentation Test

Antigenic type*	Degree of flocculation**	Supernatant turbidity†
S	++++	— or ±
S_V	+++	+
SV	++	++
V_S	+	+++
V	— or ±	++++

* S—Standard strain; S_V—intermediate strain, XII_2 cells dominant; SV—intermediate strain, XII_2 and XII_3 cells well-balanced; V_S—intermediate strain, XII_2 cells dominant; V—variant strain.

** ++++—Marked flocculation; +++—+—decreasing degrees of flocculation; ±—trace of flocculation; ——no flocculation.

† ++++—Marked turbidity; +++—+—decreasing degrees of turbidity; ±—trace of turbidity; ——no turbidity.

Procedure 3 has no advantage over procedures 1 and 2. However, it permits one to observe step by step the sedimenting effect of ammonium sulfate on the bacterial suspensions. It was the initial procedure employed in these investigations and may be of interest.

In summary, it may be pointed out that procedure 1 permits the rapid determination of the presence or absence of significant amounts of XII_2 or variant type cells in cultures of *S. pullorum*. Procedure 2, in which carefully standardized suspensions of the test organisms are used, allows one

to distinguish more concisely S, V, and I type cultures.

For routine typing, procedure 1 used alone may be satisfactory when it is not considered essential to distinguish V and I type cultures, and one wishes merely to determine the presence or absence of significant amounts of XII_3 type cells in an *S. pullorum* culture. In any case, all cultures should be typed first using procedure 1 since, in most instances, it permits the elimination of S type cultures, the most frequently encountered type, from further consideration. *Salmonella pullorum* cultures found to contain XII_3 cells by procedure 1 may be further typed, if desired, using suspensions of standardized density as directed in procedure 2.

RESULTS AND DISCUSSION

In an effort further to evaluate the procedures outlined above, strains of *S. pullorum* which had been serologically typed by other workers as S or V were requested of nine laboratories located in this country and Canada. A total of 76 serologically typed *S. pullorum* cultures was received. Approximately one half of these cultures had been designated S strains and one half V strains.

All of the cultures were typed in this laboratory using the ammonium sulfate sedimentation test, procedures 1 and 2. Sedimentation tests on all except three of the cultures were in general agreement with the previous serological tests conducted in independent laboratories. When the three cultures were again serologically typed in this laboratory, using single colony selection, the typing results were found in agreement with the sedimentation test results. Two of the three cultures were designated V types by the sender but were found to be S forms. One of the cultures had been typed S by the sender and was found to be in the V form.

Approximately one fourth of the cultures that had been designated V strains as received were found to contain XII_3 cells in large enough amounts to be classified I strains when tested by procedure 2. Some of the cultures received during this experimental study had not been previously typed. Sedimentation tests made here on these cultures of unknown antigenic balance were found in agreement with serological tests.

The motile organism, *Salmonella typhosa*, has the same somatic antigenic structure as *S. pullorum*.* Moreover, *S. typhosa* has been found by Kauffmann [2] and Edwards and Bruner [8] to exhibit a form variation in antigenic component XII identical to that of *S. pullorum*. Thus, it seemed desirable to study the sedimentation effect of ammonium sulfate on a strain of *S. typhosa* which exists in the XII_2 form and one which exists in the XII_3 form. *Salmonella typhosa* T_2 (IX, XII_1, XII_3) and *S. typhosa* 0901 (IX, XII_1, XII_2, XII_3) were obtained for this purpose.

The reaction of *S. typhosa* T_2 in ammonium sulfate solutions of varying concentrations was characteristic of S strains of *S. pullorum*. *Salmonella typhosa* 0901 reacted in the ammonium sulfate solutions in a manner characteristic of V types of *S. pullorum*. Macroscopic ammonium sulfate sedimentation test results, using suspensions of *S. typhosa* T_2 and 0901, are illustrated in table 2.

TABLE 2—Macroscopic Ammonium Sulfate Sedimentation Tests of Salmonella Pullorum Antigen Strains, Salmonella Gallinarum, and Salmonella Typhosa

Strain*	Degree of flocculation**	Supernatant turbidity†
S. pullorum 11	++++	–
S. pullorum 17	++++	–
S. pullorum 19	++++	–
S. pullorum 20	++++	–
S. pullorum 296	+	+++
S. pullorum 77	–	++++
S. pullorum 79	–	++++
S. gallinarum	++++	–
S. typhosa T_2	++++	–
S. typhosa 0901	–	++++

* Suspensions were prepared as described in the text and standardized to 100 x tube No. 1 of the McFarland scale; ** refer to table 1; † refer to table 1.

Sixty separate strains of the nonmotile fowl typhoid organism, *Salmonella gallinarum*, have been tested using the macroscopic ammonium sulfate sedimentation test. *Salmonella gallinarum* is considered to possess the same somatic antigenic components as *S. pullorum* and *S. typhosa* but, unlike these organisms, has not been found to give rise to antigenic variants. Serological studies to substantiate the absence of XII_2 antigenic forms in *S. gallinarum* cultures have been reported by Gwatkin and Dzenis, [15] Burr *et al.*,[16] Sudds and Jungherr,[17] and Marthedal.[18]

* Some *S. typhosa* strains also contain the somatic Vi antigen.

All of the 60 strains of *S. gallinarum* examined during this study were found to react to ammonium sulfate in a manner characteristic of S strains of *S. pullorum* and all suspensions were cleared by a 330-Gm./l. concentration of ammonium sulfate using either procedure 1, 2, or 3 (table 2).

Typing results on several *S. pullorum* antigen strains of known antigenic nature are presented in table 2. Strains 17, 19, 20, and BAI 11 are well-known S strains. Strains BAI 77, BAI 79, and Canadian 296 are variant strains which are widely used in the preparation of polyvalent pullorum antigen. Sedimentation tests on these cultures further confirmed their antigenic nature which has been previously established by serological methods. Canadian strain 296 was found to contain some XII_3 cells but was predominant in XII_2 forms.

It would be of interest to extend these investigations to other organisms in serological group D of the Salmonella genus which have been found to exhibit form variation in somatic antigen XII. Furthermore, the study of organisms in other serological groups of the Kauffmann-White Schema, which possess somatic antigen XII, but not IX, and which are subject to form variation in antigen XII, would be of equal interest.

SUMMARY

A new nonserological method for differentiating standard, variant, and intermediate antigenic types of *Salmonella pullorum* is presented and discussed. The method is based on the relative sedimenting effect of ammonium sulfate on the various antigenic forms of the organism, which is, in turn, dependent upon the XII_2 or variant cell content of the cultures.

An aqueous solution of ammonium sulfate, containing 330 Gm. of the salt per liter, clears standard type suspensions following a short period of incubation and centrifugation, has little effect on the turbidity of variant type suspensions, and only partially clears intermediate type suspensions.

Results of the macroscopic ammonium sulfate sedimentation test on *S. pullorum* strains obtained from independent laboratories and on stock standard and variant strains of the organism presently employed for antigen production were found in close agreement with serological typing results. The examination of 60 *Salmonella gallinarum* cultures using the macroscopic ammonium sulfate sedimentation test did not reveal the presence of variant type cells in any of the cultures.

References

[1] Williams, J. E.: Antigenic Studies Using Ammonium Sulfate. I. The Relative Sedimentation Effect of Ammonium Sulfate on the Various Antigenic Types of *Salmonella Pullorum*. Am. J. Vet. Res., *14*, (1953):

[2] Kauffmann, F.: A Typhoid Variant and a New Serological Variation in the Salmonella Group. J. Bact., *41*, (1941): 127-140.

[3] Younie, A. R.: Pullorum-like Infection of Fowl. Canad. J. Comp. Med., *6*, (1942): 172-173.

[4] Byrne, J. L.: Variant or Atypical Strains of *Salmonella Pullorum*. A Serological Study. Canad. J. Comp. Med., *7*, (1943): 227-238.

[5] Gwatkin, R.: Studies in Pullorum Disease. IV. The Effect of Bacteriophage on Regular and Variant Strains of *S. Pullorum*. Canad. J. Comp. Med., *9*, (1945): 43-45.

[6] Williams, J. E., and Jones, Florence: Unpublished data.

[7] Gwatkin, R.: Studies in Pullorum Disease. XX. Attempts to Produce Form Variation in *Salmonella Pullorum* and to Differentiate the Forms by Growth on Serum Agar. Canad. J. Comp. Med., *12*, (1948): 8-11.

[8] Edwards, P. R., and Bruner, D. W.: Form Variation in *Salmonella Pullorum* and Its Relation to X Strains. Cornell Vet., *36*, (1946): 318-324.

[9] Wright, M. L., and Edwards, P. R.: The Serologic Differentiation of *Salmonella Pullorum* Forms. Am. J. Vet. Res., *9*, (1948): 386-388.

[10] Edwards, P. R., Bruner, D. W., Doll, E. R., and Hermann, G. J.: Further Notes on Variation in *Salmonella Pullorum*. Cornell Vet., *38*, (1948): 257-262.

[11] Snoeyenbos, G. H., Crotty, A. M., and Van Roekel, H.: Some Antigenic Characteristics of *Salmonella Pullorum*. Am. J. Vet. Res., *11*, (1950): 221-225.

[12] Snoeyenbos, G. H., Bachman, B., and Van Roekel, H.: A Survey of the Incidence of Antigenic Forms of *Salmonella Pullorum* Occurring in the United States. Proc., 23rd Ann. Meet. Northeast Conf. of Lab. Workers in Pullorum Disease Control, 1951.

[13] MacDonald, A. D.: Recent Developments in Pullorum Antigen for the Rapid Whole-Blood Test. Rep. of Conf. of Nat. Poultry Improvement Plan, (1941): 122-127.

[14] McFarland, John: The Nephelometer: An Instrument for Estimating the Numbers of Bacteria in Suspensions Used for Calculating the Opsonic Index and for Vaccines. J. Am. M. A., *49*, (1907): 1176-1178.

[15] Gwatkin, R., and Dzenis, L.: Fowl Typhoid. I. Comparison of Antigenicity of Sixteen Gallinarum Antigens. Canad. J. Comp. Med., *15*, (1951): 15-20.

[16] Burr, W. E., Dondero, N. C., and Jungherr, E. L.: Experiences in the Isolation of *Salmonella Pullorum* from Serologic Reactors. Proc., 20th Ann. Meet. Northeast Conf. of Lab. Workers in Pullorum Disease Control, 1948.

[17] Sudds, Dorothy, and Jungherr, E. L.: Typing of *Salmonella Pullorum* and *Salmonella Gallinarum* Cultures Isolated in Connecticut in 1950 and 1951. Proc., 24th Ann. Meet. Northeast Conf. of Lab. Workers in Pullorum Disease Control, 1952.

[18] Marthedal, H. E.: Studies on Pullorum Disease. Serological, Cultural and Antigenic Aspects. Nord. Vet.-med., *4*, (1952): 201-224.

An Intradermal Test as a Diagnostic Aid in Newcastle Disease

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DURING THE PAST two years, a strain of Newcastle disease virus * has been modified at the Rhode Island Agricultural Experiment Station by serial skin passages in chickens. By this technique, it was possible to develop a virus strain which exhibited a low infectivity for the respiratory tract but which acquired the ability to produce extensive thickening of the skin upon intradermal inoculation into Newcastle disease-susceptible birds.

Egg propagation of the skin-adapted virus increased its infectivity for the respiratory tract and greatly enhanced its ability to produce consistent and extensive skin lesions when used intradermally. Since it was found that the skin reaction occurred only in Newcastle disease-susceptible individuals, it was felt that this agent might be used intradermally as a test to detect resistance to Newcastle disease (ND) and in this way aid in the diagnosis of respiratory infections in chickens.

In order to evaluate this hypothesis, the following experiments were undertaken.

PROCEDURE

Field Cases.—Starting Jan. 1, 1951, the intradermal testing technique was used where practicable on field cases of respiratory disease outbreaks in chickens presented for diagnosis at this laboratory. When several specimens were presented material was collected from the trachea (and lungs, if some were destroyed) for the routine virus isolation studies. In most cases, 2 surviving birds were inoculated intradermally with egg-propagated, skin-adapted, Newcastle disease virus (NDV). This virus had been modified by 23 serial skin passages before it was used to inoculate embryonating eggs. The allantoamniotic (a.a.) fluid from the first egg passage was used throughout these trials as the test agent and is designated "egg-propagated skin NDV." The intradermal inoculation was done with a tuberculin syringe fitted with a 26- or 27-gauge needle. The site of injection was the crural space, a featherless area on the lateral surface of the thigh. Approximately 0.1 cc. of the a.a. fluid was inoculated. The birds were observed on the second, third, and fourth days after inoculation and records kept of the skin reaction, herein termed "takes." A "take" consisted of an unmistakeable circumscribed, thickened, edematous area of the skin at the site of injection.

The data obtained from the intradermal testing of the various field cases are presented in table 1, along with the records of virus isolations and vaccination histories.

EXPERIMENTAL

Trial 1.—This trial was designed to determine how soon after exposure to NDV the susceptible individual would develop sufficient immunity to prevent a skin reaction following intradermal inoculation with the egg-propagated skin NDV. Correlation of the skin reaction and the onset of respiratory symptoms was also to be observed.

Two hundred 7-week-old Barred Plymouth Rock X White Plymouth Rock hybrid chicks, which had been raised in isolation from time of receipt at 1 day of age, were swabbed intratracheally with undiluted a.a. fluid containing P-24, a pathogenic strain of NDV.

Starting one day after intratracheal exposure, on successive days groups of 10 birds each were intradermally inoculated with the egg-propagated skin NDV. The groups of inoculated birds were kept in separate crates in another house and observed for takes on the second and third days following skin inoculation. All uninoculated birds were examined once daily, and when respiratory symptoms appeared the birds were identified by leg bands.

On the fourth day following intratracheal exposure, 4 birds exhibited respiratory symptoms and were included in the lot which was inoculated intradermally on that day. During the next two days, the fifth and sixth after exposure, respiratory symptoms were observed in most of the remaining birds, and on each day part of the group of 10 inoculated intradermally consisted of individuals that had been showing respiratory symptoms for less than twenty-four hours. During the next eight days, all birds intradermally tested had shown respiratory signs for at least one day.

After two weeks, skin inoculations were continued at weekly intervals using, for the most part, birds that had never shown respiratory symptoms. Table 2 shows the results of this trial.

Trial 2.—This trial was undertaken in order to obtain more information on the intradermal test-

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The authors are members of the Department of Animal Pathology, University of Rhode Island, Kingston.

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* New Jersey Roakin strain.

ing of birds on the day they first exhibit respiratory symptoms. Forty-two ND-susceptible 12-week-old Barred Plymouth Rock X White Plymouth Rock hybrids were swabbed intratracheally with Gee strain of NDV, isolated from a severe field outbreak a week earlier. These birds were examined once each day. On the day they first exhibited respiratory symptoms, they were inoculated intradermally with egg-propagated skin NDV. Between the fourth and seventh days after exposure, 39 of the 42 birds showed respiratory symptoms and were inoculated intradermally, placed in crates, and observed over a five-day period for takes. Five birds, as indicated in table 3, showed severe nervous symptoms after several days and were destroyed. Of the 3 birds which had not shown respiratory signs, 2 developed nervous symptoms in the form of lameness and/or drooping wings. The data obtained from this trial are presented in table 3.

DISCUSSION

Table 1 includes data on 35 field cases involving 74 skin-inoculated birds. In cases where takes developed following intradermal inoculation, one of the following results was obtained by virus isolation studies: *(a)* failure to isolate a virus (7 cases, 14 birds); *(b)* isolation of infectious bronchitis virus (9 cases, 21 birds); *(c)* isolation of Newcastle disease virus (2 cases, 4 birds).

From preliminary trials and the experimental work presented in this paper, it appears that the intradermal testing of birds with immunity to ND results in no clinical skin reaction, whereas the intradermal testing of ND-susceptible birds results in a skin reaction which is definitely observable the third and fourth day after inoculation.

This would mean that 2 cases in this group (5 and 16) showed an inconsistent reaction. These were the cases from which NDV ("*c*" above) was isolated. When the birds in case 5 were presented, only 1 was showing respiratory symptoms. In the

TABLE 1—Correlation of the Intradermal Testing Results with Virus Isolations and Other Data

Case No.	No. birds	Respir. symptoms at time of submission	"Takes" on NDV skin inoculation (No. birds)	Virus isolations	Vaccination record for ND		
					Not vacc.	Killed vaccine	Live vaccine
1	2	No	2	Failure	x		
6	2	Yes	2	Failure	x		
10	2	Yes	2	Failure	x		
14	2	No	2	Failure	x		
15	2	No	2	Failure	x		
17	2	No	2	Failure		x	
18	2	Yes	2	Failure		x	
8	3	Yes	3	BRV	x		
9	2	Yes	2	BRV	x		
11	3	Yes	3	BRV	x		
13	2	Yes	2	BRV	x		
19	2	Yes	2	BRV	x		
20	2	Yes	2	BRV	x		
24	2	Yes	2	BRV	x		
29	2	Yes	2	BRV	x		
31	3	Yes	3	BRV	x		
5	2	**	1	NDV	x		
16	2	Yes	2	NDV	x		
2	1	No	0	Failure	x		
32	3	Yes	0	Failure	x		
33	4	No	0	Failure	x		
34	2	Yes	0	Failure			Wing-web
35	2	No	0	Failure			Wing-web
21	2	Yes	0	BRV			*
23	2	Yes	0	BRV			Wing-web
28	2	Yes	0	BRV			Wing-web
3	2	Yes	0	NDV	x		
4	2	Yes	0	NDV		x	
7	2	No	0	NDV	x		
12	1	Yes	0	NDV	x		
22	2	Yes	0	NDV	x		
25	2	Yes	0	NDV	x		
26	2	Yes	0	NDV			Intranasal
27	2	Yes	0	NDV			Intranasal
30	2	Yes	0	NDV	x		

ND—Newcastle disease; NDV—Newcastle disease virus; BRV—bronchitis virus.
* Previously diagnosed outbreak of ND in flock; ** one of the 2 specimens was exhibiting symptoms.

other case (16), the birds had started exhibiting respiratory symptoms the day they were submitted. Since the birds in these 2 cases were evidently presented early in the disease outbreak, it is logical to assume that their immunity to ND at the time of the intradermal testing was not sufficient to prevent a skin reaction. It should also be noted that tracheal material used for virus isolation studies was collected from all specimens submitted to the laboratory and not only from the birds intradermally tested.

TABLE 2—Results of the Intradermal Testing of Birds Experimentally Exposed to Newcastle Disease Virus

Days after exposure to NDV	No. birds showing resp. symptoms at or before time of skin inoculation*	Skin reactions, 2nd day after inoculation "takes"	d***	Skin reactions, 3rd day after inoculation "takes"	d***
1	0	10		10	
2	0	10		10	
3	0	10		10	
4	4**	6		3	
5	6**	0		0	
6	7**	0		0	
7	10	0		0	
8	10	0		0	
9	10	0		0	
10	10	0		0	
11	10	0		0	
12	10	0		0	
13	10	0		0	
14	10	0		0	
21	4	0	2	0	1
28	0	0	4	0	2
35	0	0	2	0	
42	0	1	5	1	4
49	0	2	3	1	3

NDV—Newcastle disease virus. * 10 birds used per group; ** had come down with symptoms on day of skin inoculation. Subsequent groups showed symptoms for at least one day or had shown symptoms and recovered before skin inoculation; *** discoloration (yellowing or hemorrhagic) at the site of inoculation is indicated by d.

The 7 cases (14 birds) which developed takes and from which no virus could be found can not be used to evaluate the intradermal testing technique, but their skin reactions would indicate that they were susceptible to ND.

The results of the intradermal test in the other 9 field cases (21 birds) from which infectious bronchitis virus was isolated point to the diagnostic value of a test to determine individual susceptibility to ND.

In only 2 of the above cases (both virus isolation failures) was vaccination practiced, and both of these were treated with killed ND vaccine at 1 day of age.

In cases where takes did not develop after skin inoculation, one of the following findings was obtained by virus isolation studies: *(a)* failure to isolate a virus (5 cases, 12 birds); *(b)* isolation of infectious bronchitis virus (3 cases, 6 birds); *(c)* isolation of Newcastle disease virus (9 cases, 17 birds).

Here again, the cases from which no virus was isolated can not be used to evaluate the intradermal test, but the results of the experimental trials would indicate that these birds had some immunity to ND, since they failed to develop takes. Table 1 indicates that virus isolation failures were more common in cases in which the birds did not show respiratory symptoms at the time of submission. In these cases, it is often very difficult to isolate a virus and to arrive at a satisfactory diagnosis without the aid of a test to indicate immunity to ND.

Where infectious bronchitis virus was isolated, 1 case (21) had had a previously diagnosed outbreak of Newcastle disease and the other 2 cases (23, 28) had been

TABLE 3—Results of the Intradermal Testing of Birds at Onset of Respiratory Symptoms of Newcastle Disease

Days after exposure until onset of symptoms	No. birds exhibiting respiratory symptoms	"Takes"—(No. birds) Days after skin inoculation *					Remarks
		1	2	3	4	5	
4	11	0	0[d]	0	0	0	2 birds destroyed 4th day of illness due to severe nervous symptoms.
5	10	0	0	0	0	0	2 birds destroyed 3rd day due to severe nervous symptoms.
6	9	0[d]	0	0	0	0	
7	9	0	0	0	0	0	1 bird destroyed 3rd day due to severe nervous symptoms.

* Intradermal inoculations were made on day symptoms were first observed; [d] indicates discoloration at the site of inoculation in 1 bird.

immunized with live Newcastle disease vaccine by the wing-web method.

The examination of table 1 gives data that shows that, as a result of intradermal testing, the susceptibility or immunity to ND may be ascertained. This information, together with the flock history provided by the poultryman, has been of definite diagnostic value. An apparent diagnostic limitation of the test would be in flocks successfully immunized against ND or which had undergone an earlier undiagnosed outbreak of ND and currently were infected with another respiratory disease.

A study of the results of the first experimental trial (table 2) indicates that takes appeared in birds that were inoculated intradermally any time up to the onset of respiratory symptoms. Six of 10 birds skin-inoculated on the day symptoms were first observed in the pen had takes, while 4 were negative when examined two days after intradermal inoculation. On the third day postinoculation, 3 of the 6 birds that had shown takes the previous day were negative. None of the skin-inoculated birds, which had shown symptoms for at least twenty-four hours, developed takes. These results would indicate that by the time respiratory symptoms are evident in ND, a sufficient immunity has been built up in most birds to prevent the development of a skin reaction following the inoculation of the test virus. After twenty-four hours of symptoms, all birds tested had sufficient immunity to prevent the intradermal reaction.

Starting the twenty-first day after exposure, most of the birds in the skin-inoculated groups had not shown respiratory symptoms. Many of these birds developed local discoloration (yellowing or hemorrhagic) at the site of inoculation, with very little or no thickening. A number of these reactions diminished or disappeared by the third or fourth day after inoculation.

The results of the second experimental trial (table 3) substantiate the work reported in table 2. Here it was found that birds inoculated intradermally with the test agent after exhibiting respiratory symptoms did not show typical skin reactions. Two birds exhibited discoloration at the site of inoculation; however, this disappeared by the third day.

SUMMARY

The intradermal testing technique to detect individual immunity to Newcastle disease was evaluated by using it on 74 birds, representing 35 field cases of respiratory disease outbreaks in chickens which were submitted to the laboratory for diagnosis. Virus isolation studies were carried out on these cases and of the 23 instances where a virus was isolated, only 2 showed an inconsistant finding on skin testing.

In the first experimental trial where groups of susceptible birds were intradermally tested at intervals after exposure to Newcastle disease virus, it was shown that until respiratory symptoms are evident most tested birds developed a "take' indicating they had little or no immunity.

Intradermal testing of artificially infected birds on the day respiratory symptoms were first noticed (trials 1 and 2) did not cause a skin reaction that was observable on the third day after inoculation in 95 per cent (53 of 56) of the birds tested. These trials also revealed that all birds which had shown respiratory symptoms for at least one day had sufficient immunity to prevent the development of a skin reaction after intradermal exposure with the test virus.

The intradermal test presented here appears to be a convenient and accurate method in determining individual immunity to Newcastle disease, and in this way should be a definite aid in diagnosing respiratory infections of chickens.

Isolation of Capsular Polysaccharides from Colonial Variants of *Pasteurella Multocida*

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IN A PREVIOUS study, a type specific substance was extracted from *Pasteurella multocida* organisms by heating them in normal saline at 56 C. for an hour. On the basis of the precipitation of this substance, three different serological types of *P. multocida*, designated A, B, and C, were identified. The purpose of this report is to describe the method of isolation, and some of the chemical and biological properties of the soluble capsular substances.

LITERATURE REVIEW

The complex dissociation pattern of *P. multocida* has been described by Elberg and Chen-Lee Ho.[5] Carter and Byrne[4] have referred to the principal colonial variants as fluorescent (F), intermediate (I), blue (B), and mucoid (M). The source, virulence, and serological behavior of these different variants are dealt with in several recent reports.[2-4] Kendall, Heidelberger, Dawson,[6] and Seastone[12] prior to 1939 isolated serologically inactive capsular polysaccharides from mucoid group C streptococci.

In 1928, Hoffenreich[6] reported the isolation of a capsular polysaccharide from *Bacillus aviseptic*us possessing properties similar to the well-known soluble specific substances of the Pneumococcus. His polysaccharide was serologically active, had a nitrogen content of 1.2 per cent and, after hydrolysis, yielded 46 per cent reducing substances. Some years later Pirosky[9,11] recovered two distinct glycolipid antigens from "smooth" and "rough" forms of *Pasteurella aviseptica*.

MATERIALS AND METHODS

Cultures.—These were: strain 41 type A (buffalo), 312 type B (bovine), and 398 type C (canine). Culture 312 was freshly isolated from a case of calf pneumonia. The other strains of *P. multocida* have been employed in studies for a number of years.

Serological Procedures.—Rabbit serums were prepared from all cultures in the manner previously described.[3] A precipitin test was employed in which 0.1-ml. amounts of antigen dilutions were layered over 0.1-ml. amounts of undiluted antiserums. The tubes were incubated for one hour at 37 C., then read. The presence of a white precipitate in the form of a thin ring at the junction of the antigen and antiserum constituted a positive reaction.

Cultivation of Pasteurella Multocida.—Organisms were cultivated on beef infusion agar contained in oversize Petri plates (diameter 15 cm.). Thirty to 35 plates were employed for each preparation. Polysaccharides were recovered from cultures 41 and 312, but those recovered from the latter were studied in the greatest detail. It was possible by altering the constituents of the culture medium to produce cultures possessing predominantly I variants, F variants, and M dissociants.

In order to obtain the I variant, culture 312 was injected into a mouse, intraperitoneally. At fifteen to eighteen hours after injection, heart's blood was removed aseptically and a few drops were inoculated into each of several tubes of beef infusion broth. After eight to fifteen hours' incubation, each Petri plate containing beef infusion agar was seeded with 0.2 ml. of broth. The F variant was obtained in the same manner except that the beef infusion agar contained 0.3 per cent sucrose and 0.3 per cent dextrose. To produce the M variants culture, 312 was not passed in a mouse, the broth cultures being seeded direct from an agar slant. After incubation the Petri plates containing beef infusion agar supplemented with 0.3 per cent sucrose and 0.3 per cent dextrose were each seeded with 0.5 ml. of broth. The growth was washed off with cold distilled water after incubation for fifteen to eighteen hours. Great care was taken to eliminate agar from the harvest of organisms.

Isolation of Polysaccharides.—The harvested suspensions were heated in a water bath at 56 C. for one hour, after which the bacteria were sedimented by high speed (10,000 r.p.m.) centrifugation. The slightly opalescent, amber supernatant fluid was reduced to 15 to 20 ml. by vacuum distillation and treated with 3 volumes of 95 per cent ethyl alcohol containing 0.25 per cent sodium acetate. A viscous, stringy precipitate appeared which was collected by the rotation of a glass rod and/or centrifugation. The viscous precipitate was rubbed in an amount of distilled water gradually increasing to 10 ml. This was centrifuged at high speed to sediment insoluble material. The whole procedure was repeated and the final alcoholic precipitate, the third, was sedimented by centrifugation. It was then washed with absolute alcohol and dried *in vacuo* over calcium chloride. Prior to conducting chemical analyses, the polysaccharides were further dried over phosphorus pentoxide in a drying chamber at 65 C.

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RESULTS

Polysaccharides possessing different chemical and biological properties were isolated from each of the three dissociation phases, *i.e.*, the I, F, and M variants. Nine preparations of polysaccharides from culture 312 were studied. Some of their properties are presented in table 1.

TABLE 1—Properties of Polysaccharides Isolated from Different Dissociants of Bovine Culture 312, a Type B, Pasteurella Multocida

	Sources of polysaccharides Predominantly		
	I variants	F variants	M variants
Yield	14.0 mg.	34.5 mg.	43.0 mg.
Nitrogen*	5.7 %	4.9 %	5.6 %
Reducing substances**	13.6 %	35.8 %	42.7 %
Phosphorus†	4.6 %	1.89 %	1.98 %

* Micro-Kjeldahl; ** Somogyi-Shaffer-Hartmann; † Fiske-Subba Row.

The figures in regard to yield and chemical composition represent averages of the values obtained from several preparations. The differences in the values obtained from the chemical analyses of the various preparations from the same variant were in the range of 1 to 2 per cent. Solutions of the unhydrolyzed substances gave negative Fehling and biuret tests, and positive Molisch (for hexoses) and Bial (for pentoses) reactions. Reducing substances were liberated after hydrolysis with 2 N HCl in sealed ampoules in a boiling water bath for three hours. The presence of hyaluronic acid in the M substance was demonstrated by the action of hyaluronidase. The depolymerization of the polysaccharide was determined by the turbidometric method of Kass and Seastone.[7] The yield of dried bacilli remaining from each preparation after extraction was approximately 500 mg.

Physical Characteristics.—The polysaccharides of the three different variants possessed somewhat different physical characteristics, although, in general, the F and I substances were similar. In the desiccated state, the latter were white to cream in color, and flaky and horny in consistency. The dried M substance was white and consisted of a white, fluffy powder possessing numerous fine filaments. It dissolved readily in distilled water and a 0.1 per cent solution possessed a slight opalescent cast. The F and I substances in 0.1 per cent solutions were less soluble and displayed more opalescence.

Serological Specificity.—The results of the precipitation tests conducted with F and M substances are presented in table 2. Although, in some instances, there were cross reactions, a considerable degree of specificity was displayed.

Immunogenicity.—A small experiment was conducted to determine whether or not the two polysaccharides were immunogenic in mice. One group of mice (Swiss albino, 18 to 22 Gm.) was inoculated with M polysaccharide while another was injected with the F substance. Three 0.5-ml. doses of 0.01 per cent saline solution was administered intraperitoneally to each mouse. The injected and control groups were challenged nine days after the last inoculation with 0.5 ml. of various dilutions of an eighteen-hour culture of strain 312. The results are presented in table 3.

The 18 mice surviving were divided into two groups, each consisting of 9 mice. One group was rechallenged with 0.5 ml. of a 10^{-5} dilution of strain 398 type C, and the other with the same amount of culture 41 type A. Repeated titrations in mice have shown the $l.d._{50}$ of these two cultures for mice to lie between 0.5 ml. of a 10^{-8} and 0.5 ml. of a 10^{-9} dilution. All of the mice in both groups succumbed to infection. The results of these experiments indicate that the F polysaccharide is immunogenic and

TABLE 2—Precipitation Tests Demonstrating the Serological Specificity of the F Substance and the Serological Inactivity of the M Substance of Pasteurella Multocida

Serum	Polysaccharide antigen	Saline dilutions of antigens				Controls
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	
41 (F and I) Type A	41(F)*	++**	+†	+	+	−
	312(F)	+	−	−	−	
	312(M)	−	−	−	−	
312 (F and I) Type B	41(F)	−	−	−	−	−
	312(F)	++	+	+	+	
	312(M)	−	−	−	−	
398 (F and I) Type C	41(F)	+	−	−	−	−
	312(F)	+	−	−	−	
	312(M)	−	−	−	−	
312 (M) Type B	312(M)	−	−	−	−	−
	312(F)	+	−	−	−	−

* Variant source; ** very marked ring; † distinct ring.

TABLE 3—Immune Response in Mice to Injections of the M and F Polysaccharides of Pasteurella Multocida as Indicated by Homologous Challenge

Injected with	Deaths per challenge dilution group			
	10^{-5}	10^{-6}	10^{-7}	10^{-8}
M substance (strain 312)	5/5	.....	5/5	4/5
F substance (strain 312)	1/7	0/6	0/6	.....
Control groups	5/5	5/5	5/5	4/5

Numerator = died; denominator = number challenged.

immunologically specific. The M substance elicited no appreciable immune response in the above experiment.

Toxicity.—Doses of the M and F polysaccharides (culture 312) in amounts varying from 0.5 to 3.0 mg. were inoculated intraperitoneally into adult mice. There was no reaction other than evidence of considerable abdominal distress for several hours after inoculation.

DISCUSSION

The values obtained for reducing substances and nitrogen differ considerably with those reported by Hoffenreich. However, a perusal of chemical data on various bacterial polysaccharides discloses considerable variation in the results obtained by different workers studying the same bacterial species by different methods.[1]

The glycolipid antigens of *P. aviseptica* isolated by Pirosky [9,11] appear to be distinct from the polysaccharides described in this study. That they are somatic antigens only is strongly suggested by their chemical nature and the fact that they were isolated by the technique employed widely by Boivin for the isolation of the well-known somatic glycolipid antigens. These "Boivin antigens" of *P. aviseptica* differ also from the capsular polysaccharides in that they were highly toxic, the $l.d._{50}$ for mice being in the range of 0.05 to 0.1 mg.

Pirosky [9] implied that *P. aviseptica* did not possess a capsular antigen distinct from the glycolipid antigen. Such an inference may be attributed to the fact that his cultures were probably in an acapsular or blue phase as is often the case with old laboratory strains. That such was the case is suggested by his reference [10] to the avirulence for mice of his challenge culture.

Strains of *P. multocida* isolated from acute pasteurellosis usually produce many F colonies and a few I variants. It would appear that a preponderance of I variants rather than F forms was produced on beef infusion agar because of a limited supply of carbohydrate. That the difference between the F and I variants may be more quantitative than qualitative in nature is suggested by the fact that both substances displayed marked serological specificity. An excess of carbohydrate and surface moisture appeared to exert a selective action for M variants.

The results of experimental work conducted on *P. multocida* in past years should be reviewed critically in the light of recent observations involving different serological types, dissociation pattern, and antigenic structure. Considering that the evidence for the value of hemorrhagic septicemia bacterins is somewhat tenuous, efforts should be made toward preparing these agents in accordance with recent serological and immunological findings. Of particular significance is the demonstration of the high immunogenicity of the capsular polysaccharide of the F variants.

SUMMARY

Capsular polysaccharides possessing different chemical, physical, and biological properties have been isolated from the F, I, and M variants of *Pasteurella multocida*. Data are presented relative to yields obtained, percentage composition of nitrogen, reducing substances, and phosphorus. Hyaluronic acid was identified in the M substance.

The F and I soluble substances were highly active and specific serologically, whereas the M polysaccharide was relatively inactive serologically. The M substance elicited little or no immunity in mice but the F substance was markedly immunogenic. Both substances were nontoxic for mice in doses up to 3 mg.

References

[1] Burger, M.: Bacterial Polysaccharides. 1st ed. Charles C. Thomas, Springfield, Illinois, 1950.

[2] Carter, G. R.: The Type Specific Capsular Antigen of *Pasteurella Multocida*. Canad. J. M. Sci., *30*, (1952): 48-53.

[3] Carter, G. R.: Some Comments on Pasteurellosis. Canad. J. Comp. Med., *16*, (1952): 150-152.

[4] Carter, G. R., and Byrne, J. L.: A Serological Study of the Hemorrhagic Septicemia Pasteurella. In press.

[6] Elberg, S. S., and Cheng-Lee Ho: Studies on Dissociation in *Pasteurella Multocida*. J. Comp. Path. and Therap., *60*, (1950): 41-50.

[7] Hoffenreich, F.: Kapsesubstanz aus *Bacillus Aviseptieus*. Centralbl. f. Bakt. Abt. Originale, *108*, (1928): 87-89.

[8] Kass, E. H., and Seastone, C. V.: The Role of the Mucoid Polysaccharide (Hyaluronic Acid) in the Virulence of Group A Hemolytic Streptococci. J. Exptl. Med., *79*, (1944): 319-330.

[9] Kendall, F. E., Heidelberger, M., and Dawson, M. H. A.: Serologically Inactive Polysaccharide Elaborated by Mucoid Strains of Group A Hemolytic Streptococcus. J. Biol. Chem., *118*, (1937): 61-69.

[9] Pirosky, I.: Sur l'Antigéne Glucido-lipidique des Pasteurella. Compt. rend. Soc. de biol., *127*, (1938): 98-100.

[10] Pirosky, I.: Sur les Propriétés Immunisantes Antitoxiques et Anti-infectieuses de l'Antigéne Glucido-lipidique de Pasteurella aviseptica. Compt. rend. Soc. de Biol., *127*, (1938): 966-969.

[11] Pirosky, I.: Sur l'Existence, chez les variants Smooth et Rough d'une Souche de *Pasteurella aviseptica*, de Deux Antigénes Glucido-lipidiques Serologiquement Distincts. Compt. rend. Soc. de Biol., *128*, (1938): 346-347.

[12] Seastone, C. V.: The Virulence of Group C. Hemolytic Streptococci of Animal Origin. J. Exptl. Med., *70*, (1939): 361-378.

Studies on Mink Food Experimentally Inoculated with Toxin-Free Spores of *Clostridium Botulinum* Types A, B, C, and E

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BOTULISM is recognized as a serious problem when it occurs in mink on large ranches. Usually, the disease is identified by its clinical manifestations without laboratory diagnostic procedures to determine the specific type of toxin causing the outbreak. In some instances, unsuccessful attempts have been made to determine the type of toxin. Before control measures may be instituted, it is necessary to know the types involved. The purpose of this investigation was to measure the susceptibility of mink to graded oral doses of the various types of toxin which were tested by intraperitoneal injections into mice and by oral injection into monkeys. As the study progressed, it became apparent that type C was etiologically significant. Following this observation a study was made of the growth and toxin production of the spores of *Clostridium botulinum* in mink food. Since type C was implicated, the role of insect larvae with which this organism was historically associated was also studied.

LITERATURE CITED

Quortrup and Holt[6] emphasized the importance of type C and demonstrated that mink when fed type C toxin were highly susceptible. In a personal communication,* Dr. Quortrup made the following statement: ". . . I have been able to kill minks with three *Cl. botulinum* strains of type A. The action of type A, however, is much slower than type C, requiring from sixty-eight to 192 hours to kill using tremendous doses (50,000,000 mouse m.l.d.'s or over) compared with type C which killed my mink in from eighteen to thirty-three hours. . . ." In a later letter,** Dr. Quortrup reported killing a mink in thirty hours with a very potent toxin of type B.

Dinter and Kull[2] described three food poisoning outbreaks due to type C on mink farms in different parts of Sweden. In a later paper covering toxicological studies during the year 1951, Kull and Moberg[7] reported that botulism was diagnosed in 13 out of 22 cases of food poisoning. Toxin of type C was proved in 12 cases, whereas, in 1 case, the examination could not be followed up because of lack of a sufficient amount of toxin-containing specimens. Monovalent type C toxoid was administered to 45,000 mink on approximately 60 farms in Sweden during 1951 and was found to be highly effective. Moll and Brandly[8] found that mink developed botulism when fed 20 to 80 million mouse m.l.d.'s (intraperitoneally) as purified toxins of both types A and B, *Cl. botulinum*.

Type C *Cl. botulinum* was first isolated by Bengtson[1] from larvae of *Lucilia caesar*,*** *Lucilia illustrus*,† commonly known as the green bottle fly, is seen during warm weather swarming about proteinaceous matter.[4]

In outbreaks of type C *Cl. botulinum* poisoning in wild ducks, large numbers of insects have been observed in muddy areas where the ducks have died.[9] Of the insects, *L. illustris* has been commonly identified. Kalmbach and Gunderson[9] reported that "Positive results were obtained on numerous occasions with soil or mud in duck-sickness areas. Often such material was heavily laden with miscellaneous organic matter, including strands of filamentous algae, masses of living and dead Lemna, copepods, snails, and insects. Debris from the Bear River flats, composed almost entirely of insect remains, disclosed the organism. Likewise, it was found in both the larvae and pupae of hydrophilid beetles, the cocoons of which studded the surface of the dense algal beds at Tule Lake. Colonies of copepods, sacrophagid larvae (dead or alive) found on or near the bodies of dead birds, and even water obtained from the vicinity of such materials disclosed the organism."

These studies would suggest that perhaps insects play a role in the dissemination of the spores and provide conditions suitable in mink food and litter for the growth and toxin production of the type C organism. The seasonal incidence of the disease in mink also may be explained on this basis.

Bengtson[1] fed a *Macaca mulatta* a 4 to 5 Gm. meat culture of type C *Cl. botulinum* on bread and the animal died of botulism in seventy-one hours. Gunnison and Meyer[5] demonstrated that monkeys were readily intoxicated by subcutaneous injection of types C and D toxin, but they resisted large doses of the same toxin *per os*. Any discrepancies in the literature may be explained by the potency of the toxin.

Dolman *et al.*[3] stressed the importance of fish in outbreaks of human botulism where type E *Cl. botulinum* was responsible.

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* Letter to Dr. Dack dated February 5, 1948.

** Letter to Dr. Dack dated February 27, 1948

*** *Lucilia caesar* is referred to as *Lucilia illustris* in North America.

† We are indebted to Dr. Robert M. Lewert of the Department of Bacteriology and Parasitology, University of Chicago, for help with the entomological aspects of this work.

MATERIALS AND METHODS

The supernatant fluids from beef heart cultures inoculated with toxin-free spores of *Cl. botulinum*, types A, B, C, or E, and incubated from five to nine days at 30 C. were fed to mink. The toxicity of each culture was determined by mouse titration. A mouth gag was used and the toxin sample was given with a syringe through a No. 14 French catheter, the tip of which was inserted into the stomach. In the case of types C and E, feeding experiments were also carried out on *Macaca mulatta*.

Following the feeding experiments, the role of the food as a medium for the growth and toxin production of *Cl. botulinum* was investigated. Frozen mink food consisting of:

Ingredients	*Percentage*
Horse meat	14.0
Fish	28.0
Beef livers (telangiectatic)	2.1
Duck offal	14.0
Kellogg cereal	8.4
Tomato pulp	2.1
Brewer's yeast	1.4
Water	30.0

was obtained in 6-lb. containers from a large mink ranch. The mixture was ground and processed in a sausage mill, frozen, and kept in storage until feeding. Forty-gram samples of the thawed food were inoculated with approximately 1 million per gram of viable toxin-free spores of *Cl. botulinum* types A, B, and C. Since the spore harvest from type E was poor, approximately 10,000 toxin-free type E spores per gram were inoculated. The spore suspensions were heated at 80 C. for ten minutes prior to inoculation to destroy the vegetative cells and the toxin.

Experiments were carried out inoculating mink food with spores of type C *Cl. botulinum* and with larvae of *L. illustris*. To obtain fly larvae, portions of mink food were placed beside a garbage can, in a stable, and in a room where mink were kept. By the end of one day, eggs were deposited on the meat portions placed near the garbage can and in the mink room. The number of flies raised was small; therefore, we supplemented our supply of experimental flies by capturing some adults in a room where raw meat was exposed.

RESULTS

Feeding Experiments.—The data in table 1 show that mink were resistant when fed types A, B, and E *Cl. botulinum* toxin and were relatively susceptible to oral doses of type C toxin.

Macaca mulatta were similarly fed types C and E toxin and the results are shown in table 2. From these studies, it is obvious that monkeys are susceptible to both types C and E toxins of *Cl. botulinum*.

Mink Food as a Medium for the Growth and Toxin Production of Cl. Botulinum.—Mink food samples were inoculated with toxin-free *Cl. botulinum* spores and incubated at 30 C. for twenty-four hours. During incubation, these samples developed large amounts of gas and odor of fermentation but not putrefaction. Samples were weighed into sterile centrifuge tubes, diluted with sterile saline solution, thoroughly mixed, and centrifuged for fifteen minutes at high speed to sediment the larger particles. The supernatant fluids were injected intraperitoneally into mice in 0.5-ml. amounts. All mice remained well, indicating that botulinum toxin was not present in the dilutions tested with all four

TABLE 1—Reaction of Mink to Oral Doses of Four Types of Clostridium Botulinum Toxin

Mink (No.)	Culture (No.)	Toxin (type)	Amount fed (ml.)	Intraperitoneal m.l.d. in mice	Results
1	T	A	10.0	1,000,000	No symptoms during 13 days of observation.
2	I	B	10.0	10,000	No symptoms during 13 days of observation.
3	CI*	C	9.0	900,000	Died in 5½ hours.
4	CI*	C	1.0	100,000	Died in less than 22 hours.
5	CI*	C	0.1	10,000	Symptoms in 24 hours; died in less than 48 hours.
6	CI*	C	0.1	10,000	Died in less than 22 hours.
1	CI*	C	0.01	1,000	Died in less than 22 hours.
2	CI*	C	0.01	1,000	Became slightly ill on 2nd day, but recovered completely.
9**	CI	C	10.0	100,000	Died in 26 hours.
11**	CI	C	10.0	100,000	Died in less than 24 hours.
12**	CI	C	10.0	100,000	Died in less than 24 hours.
7	VH	E	10.0	10,000	No symptoms during 18 days of observation.
8	VH	E	10.0	10,000	No symptoms during 18 days of observation.
9	E6	E	10.0	1,000	No symptoms during 11 days of observation.
10	E6	E	10.0	1,000	No symptoms during 11 days of observation.
11	E8	E	10.0	10,000	No symptoms during 11 days of observation.
12	E8	E	10.0	10,000	No symptoms during 11 days of observation.

* Toxic culture received from Dr. W. I. Jensen, Johns Hopkins University, Baltimore, Md.
** No symptoms developed when previously fed a toxic culture of type E.
m.l.d.—minimum lethal dose.

TABLE 2—Feeding of Macaca Mulatta with Clostridium Botulinum Toxin Types C and E

Monkey (No.)	Culture (No.)	Toxin (type)	Amount fed (ml.)	Intraperitoneal m.l.d. in mice	Results
735	CI	C	10.0	100,000	Died in less than 18 hours.
736	CI	C	1.0	10,000	Died in less than 42 hours.
650	CI	C	0.1	1,000	No symptoms during 10 days of observation.
734	CI	C	0.1	1,000	No symptoms during 10 days of observation.
618	CI	C	0.01	100	No symptoms during 10 days of observation.
617	CI	C	0.01	100	No symptoms during 10 days of observation.
707	E6	E	10.0	1,000	Died in less than 18 hours.
677	E6	E	1.0	100	No symptoms during 10 days of observation.
618	E6	E	0.1	10	No symptoms during 10 days of observation.
738	E8	E	10.0	10,000	Died in less than 18 hours.
650	E8	E	1.0	1,000	No symptoms during 10 days of observation.
617	E8	E	0.1	100	No symptoms during 10 days of observation.

m.l.d.—minimum lethal dose.

types of *Cl. botulinum*. Toxin developed in some of the control tubes of beef heart medium which were similarly inoculated with toxin-free spores and incubated at 30 C. for twenty-four hours. The results of the control tests are given in table 3.

Effect of L. Illustris Larvae on the Growth and Toxin Production of Type C Cl. Botulinum.—Since the habitat of *L. illustris* larvae is decomposed meats, the question arose as to the effect of the larvae of this insect on the growth and toxin production of spores of type C *Cl. botulinum* experimentally inoculated into mink food.

Two experiments using *L. illustris* larvae were conducted. In the first experiment, there were nine samples (table 4). For controls, mink food (sample 1) and lean beef (sample 5) were used. Three-day-old larvae that had been feeding on mink food were transferred to mink food (sample 3) and lean beef (sample 7) previously inoculated with type C spores. For the egg inoculation of mink food (sample 4) and lean beef (sample 8) containing spores, the two samples were put into a cage with the flies, and the cage was placed in an incubator at 30 C. for twenty-four hours before the first test samples were taken. Tubes containing beef heart medium were inoculated with the same level of toxin-free spores as the meat samples. Mouse titration of the supernatant fluid from the beef heart medium was used to determine the activity of the type C *Cl. botulinum* culture used. All inoculated samples contained 100,000 spores per gram. The spores were previously heated at 80 C. for ten minutes to destroy the vegetative cells and toxin. The inoculated samples were thoroughly mixed and then all samples were incubated at 30 C. After incubation, samples were weighed into sterile centrifuge tubes, diluted with sterile 0.9 per cent NaCl solution, and mixed thoroughly. Before mouse inoculations were made, each sample was centrifuged at high speed for ten minutes; then, a portion of the supernatant fluid was withdrawn. Appropriate dilutions were made when necessary. For each test sample, 2 mice were inoculated intraperitoneally with dilutions to establish an end point. The first mouse was injected with the test sample only; the second, with the test sample and 0.1 ml. of type C antitoxin. If an unprotected mouse lived more than four days, the sample was considered nontoxic at that concentration. An overheating of the incubating samples occurred between the first and third days; therefore, the results of the first experiment, shown in table 4, are for the 24-hour sampling period only.

TABLE 3—Potency of Each of Four Types of Clostridium Botulinum Toxin Produced in Beef Heart Medium After Twenty-four Hours of Aërobic Incubation at 30 C.

Culture (No.)	Type (No.)	Inoculum toxin-free spores/gram	Intraperitoneal m.l.d. in mice
T	A	1,000,000	10,000
I	B	1,000,000	less than 2
CI	C	1,000,000	100
VH	E	10,000	less than 2

m.l.d.—minimum lethal dose.

The second experiment was similar to the first except for the following (table 5): Two additional samples consisting of larvae only (sample 9 from the lean beef and sample 10 from the mink food) were taken at some of the test intervals. Sample 11 was the beef heart control. All inoculated samples contained 8,000 spores per gram. In this experiment, test samples were taken

at the end of the first, second, third, fifth, seventh, and tenth day of incubation at 30 C. No samples of mink food larvae were taken until the end of three days of incubation. The larvae (samples 9 and 10) were crushed during the mixing of these samples. The results of the mouse inoculations and the pH of the three-day samples are recorded in table 5.

TABLE 4—Mink Food and Lean Ground Beef Experimentally Inoculated with Spores of Type C Clostridium Botulinum and with Larvae or Eggs of Lucilia Illustris, and Tested After Twenty-four Hours at 30 C. for Toxin by Intraperitoneal Injection into Mice

Sample	m.l.d. In mice
Mink food	< 10
Mink food + spores	< 10
Mink food + spores + 3-day larvae from mink food	< 5
Mink food + spores + fly eggs	< 10
Ground lean beef	< 10
Ground lean beef + spores	10,000
Ground lean beef + spores + 3-day larvae from mink food	25,000
Ground lean beef + spores + fly eggs	10,000
Beef heart medium + spores	1,000

m.l.d.—minimum lethal dose.

DISCUSSION

There is a paucity of information concerning the types of *Cl. botulinum* responsible for naturally occurring outbreaks of botulism in mink. Type E might be expected to affect mink since fish offal or fish not included in the diet of man constitute an important part of the diet of these animals. Dolman *et al.*[3] have pointed out that toxin may be produced in fresh herrings experimentally inoculated with the spores of type E *Cl. botulinum* within seventy-two hours when stored at 23 C. Experimentally, however, mink appear to be resistant when fed large doses of type E toxin. Similarly, they are resistant to oral administration of large doses of types A and B toxin. In contrast to these types, mink are highly susceptible when fed toxic cultures of type C.

Probably the most significant observation in the experiments, in which *L. illustris* larvae were present in the inoculated meat cultures, was the differences between the mink food and the lean beef as a medium for type C *Cl. botulinum* spores. In the first of these experiments, the inoculated lean beef samples contained 1,000 to 5,000 times as much toxin as the corresponding inoculated mink food samples. The magnitude of the differences observed in the second experiment were similar, starting with the three-day test samples. The longer the inoculated samples were allowed to incubate (up to seven days), the greater was the potency of the toxin pro-

TABLE 5—Mink Food and Lean Ground Beef Experimentally Inoculated with Spores of Type C Clostridium Botulinum and with Larvae or Eggs of Lucilia Illustris, and Incubated at 30 C. and Tested for Toxin by Intraperitoneal Injection into Mice

	m.l.d. in mice						
Sample	1 day	2 days	3 days	5 days	7 days	10 days	pH of 3-day sample
Mink food	< 10	< 10	< 10	< 10	< 10	< 10	4.50
Mink food + spores	< 10	< 10	< 10	< 10	< 10	< 10	4.50
Mink food + spores + three-day larvae	< 10	< 10	< 10	< 10	10	< 10	4.80
Mink food + spores + fly eggs	< 10	< 10	< 10	< 10	100	100	4.95
Ground lean beef	< 10	< 10	< 10	< 10	< 10	< 10	7.60
Ground lean beef + spores	2,500	2,500	10,000	25,000	25,000	25,000	7.10
Ground lean beef + spores + three-day larvae	2,500	10,000	25,000	25,000	100,000	100,000	7.53
Ground lean beef + spores + fly eggs	2,500	10,000	25,000	100,000	100,000	100,000	8.35
Fly larvae from ground lean beef	2,500	25,000	10,000	10,000	2,500	1,000	
Fly larvae from mink food	Not tested	Not tested	< 25	< 25	< 25	Not tested	
Beef heart medium + spores	1,000	2,500	10,000	25,000	25,000	25,000	

m.l.d.—minimum lethal dose.

duced. The toxin levels were the same at ten days as at seven days, except for the larvae samples from the lean beef which seemed to steadily decrease in toxicity after the fifth day. It is quite evident that, when larvae were present in the lean beef, more toxin was produced than with the spores alone; this was true to a lesser extent with the mink food samples. The larvae themselves (sample 9) taken at various times from the inoculated lean beef (sample 8) contained a relatively large amount of toxin. At the earlier sampling intervals, the toxin level in the larvae was very similar to the toxicity of the meat-larvae mixture, but at the later analyses the toxin was at a lower level than in the meat from which the respective samples were taken.

The pH results indicate that the larvae contributed to the increased alkalinity of the mediums due to their excretion of alkaline metabolic products. It apparently required only a short period of incubation at 30 C. for the other organisms present in the mink food to lower the pH from 6.2 to a level that was inhibitory to type C *Cl. botulinum* spores. This was undoubtedly the major factor contributing to the extremely low level of toxin produced in this medium, even after incubation for as long as ten days.

From these studies, the importance of sanitation is evident. Cages should be kept scrupulously clean especially in the hot months of the year and food not eaten should be removed. Thus, fly larvae would not have sufficient time to develop between feeding intervals.

Immunization of mink with the toxoid of type C *Cl. botulinum* appears feasible in the control of botulism in mink ranches and is supported by the work of the Swedish investigators.[7] Treatment of cases should be limited to the use of monovalent type C antitoxin.

Summary

This study concerns experiments designed to elucidate the problem of botulism on mink ranches. Mink were fed toxic cultures of types A, B, C, and E *Clostridium botulinum*. In the doses of toxins fed, mink were resistant to types A, B, and E but susceptible to type C. Samples of mink food prepared at a particular ranch were inoculated with toxin-free spores of types A, B, C, and E *Cl. botulinum*. In these food samples, incubated at 30 C. for twenty-four hours and subsequently tested in mice, toxin did not develop. Control beef heart medium similarly inoculated became toxic in twenty-four hours with types A and C but not with types B and E.

Macaca mulatta were found susceptible to feeding of toxic cultures of types C and E *Cl. botulinum*.

Mice were injected intraperitoneally with the supernatant fluid from samples of mink food and ground lean beef inoculated with type C *Cl. botulinum* spores plus *Lucilia illustris* fly larvae or eggs. Lean beef was found to be a much more favorable medium for type C toxin production than mink food. The role of *L. illustris* larvae has been clearly demonstrated as being a favorable factor for growth and toxin production of type C *Cl. botulinum* when the spores were experimentally inoculated into lean beef and mink food.

References

[1] Bengtson, I. A.: Preliminary Note on a Toxin-Producing Anaërobe Isolated from the Larvae of *Lucilia Caesar*. Pub. Health Rep., *37*, (1922): 164-170.

[2] Dinter, Z., and Kull, K. E.: Ergänzende Untersuchungen uber den Botulismus beim Nerz. Nord. Vet.-med., *3*, (1951): 297-311.

[3] Dolman, C. E., Chang, H., Kerr, K. E., and Shearer, A. R.: Fish-Borne and Type E Botulism: Two Cases Due to Home-Pickled Herring. Canad. J. Pub. Health, *41*, (1950): 215-229.

[4] Graham-Smith, G. L.: Flies in Relation to Disease. Cambridge Univ. Press, (1913): 25-26.

[5] Gunnison, J. B., and Meyer, K. F.: Susceptibility of *Macacus Rhesus* Monkeys to Botulinum Toxin Type B, C, and D. Proc. Soc. Exptl. Biol. and Med., *26*, (1928): 89-90.

[6] Kalmbach, E. R., and Gunderson, M. F.: Western Duck Sickness: A Form of Botulism. Tech. Bull. 411, U. S. Dept. of Agric. 1934.

[7] Kull, K. E., and Moberg, K.: Die Häufigkeit des Botulismus bei Futtervergiftungen des Nerzes in Schweden 1951. Nord. Vet.-med., *4*, (1952): 771-775.

[8] Moll, T., and Brandly, C. A.: Botulism in the Mouse, Mink, and Ferret with Special Reference to Susceptibility and Pathological Alterations. Am. J. Vet. Res., *12*, (1951): 355-363.

[9] Quortrup, E. R., and Holt, A. L.: Case Report on Botulism Type C in Minks. J.A.V.M.A., *97*, (1940): 167-168.

Ten Constituents of the Blood Stream of Well-Fed White Rats, Chickens, Swine, Sheep, and Horses in Guatemala

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COUNTRIES of the American tropics do not produce enough animal products for human consumption. Although the importation of purebred livestock from the United States and Canada is increasing, a large number of the imported animals die and those that survive do not produce as much milk, meat, and eggs as they should. Squibb *et al.*[12] point out that this situation applies particularly to dairy cattle.

Some of the reasons for the low production and high mortality among purebred animals in these areas have been described by Work.[16] It is generally agreed that malnutrition—the result of poor practices of animal management, shortage of feed during dry seasons, and lack of technical knowledge about the feeding of farm animals—is one of the most serious problems. A fundamental approach to the problem lies in a study of blood constituents of the animals. Becker and Smith,[1] Maynard,[8] and Rusoff and Piercy [10] have discussed the usefulness of blood data in evaluating the physiological status of farm animals. Squibb *et al.*[12] have found dairy cattle in three different areas of Guatemala to have extremely low levels of calcium and phosphorus in their blood.

Levels of essential blood constituents have not been established for most animals in tropical America. This report presents data for 24 white rats, 80 chickens, 60 pigs, 13 sheep, and 10 horses. Dietary histories of all were fairly well known; the chickens and livestock had been subjected to farm management of the kind that is practiced by better farmers in Guatemala.

PROCEDURE

Experimental Animals and Diets.—The white rats used were progeny of stock that had been obtained from the Animal Husbandry Division of the United States Department of Agriculture. Equal numbers of males and females 10 weeks old were studied. They were housed in individual wire cages and had been fed the following diet *ad libitum* over a 6-week period: casein, 20.0 per cent; sucrose, 63.7; minerals, including minor elements, 4.0; corn oil, 5.0; brewers' yeast, 7.0; and cod liver oil,* 0.3.

The chickens were New Hampshire Reds—40 hens and 40 baby chicks. The hens had been obtained from eggs imported from Texas and hatched at the Instituto Agropecuario Nacional. They were kept on a green pasture of Kikuyu grass *(Pennisetum clandestinum)* and had received the following ration over a six-month period: corn, 42.5 per cent; sesame oil meal, 34.4; rice bran, 10; wheat bran, 10; minerals, including trace elements, 3.2; and delsterol.** The chicks were the progeny of the hens. They were housed in all-metal-wire cages and were fed the following rations *ad libitum* over a six-week period: ground yellow corn, 31.2 per cent; sesame oil meal, 15; corozo oil meal, 30; cottonseed oil meal, 15; dried forage meals, 5; minerals, including the minor elements, 3; and vitarich, 0.8.†

The rats and chickens were housed in the animal nutrition laboratories of the Instituto's station at Guatemala City (elevation, 5,000 ft.; average annual rainfall, 40 in.).

The swine, Duroc-Jersey weanling pigs, were kept at the Instituto's substation in Chocolá (elevation, 2,500 ft.; average annual rainfall, 177 in.). At the time blood samples were collected, the pigs had been on green pasture approximately six weeks. The pasture had been supplemented with the following rations: corn, 50 per cent; sesame oil meal, 40; minerals, including trace elements, 3; and wheat bran, 7.

The sheep, which were native (or "criollo"), ewes approximately 18 months old, were part of the Instituto's flock located at the substation Labor Ovalle in Quezaltenango (elevation, 8,000 ft.; average annual rainfall, 65 in.). At the time of blood sampling, they had been on green pasture eight weeks; pasture had been supplemented with corn, 60 per cent; wheat bran, 30; cottonseed meal, 7; and minerals, including trace elements, 3.

The horses were criollo mares 3 to 8 years old with foals 3 to 4 months old. They were located at the United Fruit Co.'s farm at Tiquisate (ele-

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* The cod liver oil contained 1,700 I.U.'s of vitamin A and 400 I.U.'s of vitamin D per gram.

** Delsterol is a DuPont product. It was fed at the rate of 0.02 per cent of the ration.

† Vitarich is a product of the Thompson Hayward Co.

vation, 100 ft.; average annual rainfall, 110 in.). They had been on green pasture approximately eight weeks at the time the blood samples were collected.

Collection of Blood Samples.—Approximately 1 to 5 ml. of blood was obtained from the rats and chicks by heart puncture.

The hens were bled from the wing veins. Approximately 2 ml. of blood was collected and permitted to coagulate, and an additional 1 ml. was collected in tubes containing an anticoagulant composed of 6 per cent ammonium oxalate and 4 per cent potassium oxalate.

The swine were bled from the tails. Approximately 5 ml. of blood was collected and permitted to coagulate, and an additional 2 ml. was collected in tubes containing the same anticoagulant.

The horses and sheep were bled by jugular venipuncture. Approximately 6 ml. of blood was collected in tubes and permitted to coagulate.

All blood samples were refrigerated immediately after being taken and were delivered to the laboratory for analysis within six hours. The blood serum of these samples was analyzed for total proteins,[6] riboflavin,[4] vitamin A and carotenoids,[3] total tocopherols,[3] alkaline phosphatase,[2] and ascorbic acid.[5, 7] The methods for ascorbic acid were modified by using a solution of copper sulfate and thiourea instead of norite as a reducing agent. The analyses of the red components of the blood were made by standard methods.[16]

RESULTS AND DISCUSSION

Data were obtained on the levels of ten blood constituents (table 1). The animals studied were healthy, well fed, and well managed. Although some knowledge of the dietary histories was available, the data are as yet too limited to permit the correlation of the observed blood values with data on production or environment.

It is apparent that variability of levels of certain nutrients in the blood will need to be considered when critical nutrition experiments are undertaken. The New Hampshire hens showed extremely variable levels of riboflavin, carotenoids, vitamin A, and total tocopherols. Such variations are not peculiar to this area: Scrimshaw *et al.*[11] have found similar variations in serum riboflavin, carotenoids, and ascorbic acid in hens in New York. The rats, baby chicks, sheep, and horses had fairly uniform values for all the blood constituents studied except vitamin A. The Duroc-Jersey pigs had comparatively uniform values for all the constituents.

It has been pointed out that malnutrition is one of the most pressing problems in tropical America. Although the increased use of blood data could do much to define the responsible deficiencies, there are as yet too few data to tell what may be considered normal levels for the several constituents reported here. It is hoped that

TABLE 1—Several Blood Constituents of White Rats, New Hampshire Red Chicks and Hens, Duroc-Jersey Swine, and Native Sheep and Horses*

Constituent	Rats (24)	Chicks (40)	Hens (40)	Pigs (60)	Sheep (13)	Horses (10)
Serum						
Total proteins (Gm./100 cc.)	6.26 ± .06	3.86 ± .04	4.98 ± .07	6.81 ± .05	7.16 ± .11	6.94 ± .16
Riboflavin (μg./100 cc.)	2.44 ± .09	.85 ± .05	69.0 ± 6.0	2.99 ± .07	.82 ± .11	1.16 ± .10
Ascorbic acid (mg./100 cc.)	1.05 ± .08	2.23 ± .07	2.45 ± .05	2.04 ± .05	1.30 ± .07	1.30 ± .13
Carotenoids (μg./100 cc.)	2.0 ± .1	867.0 ±50.0	142.0 ±16.0	7.5 ± .43	9.5 ± .7	9.7 ± 1.2
Tocopherols (mg./100 cc.)	0.01 to 0.70	0.04 to 1.91	**	.14 ± .01	.17 ± .01	.30 ± .04
Alkaline phosphatase (millimoles/liter/hour)	11.05 ± .70	.....	17.40 ± .92	2.05 ± .06	1.97 ± .11	9.10 ± .67
Vitamin A (μg./100 cc.)	24.4 ± 1.4	53.4 ± 2.2	32.0 ± 3.3	25.2 ± .64	33.8 ± 2.0	12.1 ± 1.23
Whole Blood						
Hemoglobin (Gm./100 cc.)	13.8 ± .2	5.6 ± .1	6.7(20) ± .15	11.2(30) ± .12	†	†
Hematocrit (%)	44.0(3)	29.0 ± .3	31.0(20) ± .5	37.6(30) ± .38	†	†
Red cell count (thousands per cu. cm.)	6,780.0 ±100.0	2,280.0 ±50.0	2,445.0(20) ±74.0	6,545.0(30) ±107.0	†	†

* Figures in parentheses refer to the number of animals sampled; figures after the ± signs are standard errors of the mean.

** 20 hens had 0.00 mg. total tocopherols per 100 cc. of serum; the rest ranged from 0.01 to 0.35 mg./100 cc. Standard error for 22 hens was 0.10 ± 0.02 mg./100 cc.

† These values were not determined. Becker and Smith[1] give the following report for sheep: hemoglobin, Gm. 70, 12.4 Gm./100 cc.; hematocrit, 37.9/100 cc.; and red cell count, 11.9 ± 1.2 million per cubic centimeter.

the figures in the table may serve as reference or base line data upon which to compare animals fed similar or diffierent diets, and that they may serve as a basis for controlled feeding experiments in the future.

SUMMARY

Means with standard errors are given for the following constituents in the blood stream of rats, hens, and baby chicks, pigs, sheep, and horses: total proteins, riboflavin, ascorbic acid, carotenoids, tocopherols, alkaline phosphatase, vitamin A, hemoglobin, hematocrit, and red cell count.

These data may serve to compare with data for other animals fed similar or different diets. The potential value of blood data as an aid in evaluating the physiological status of farm animals in the American tropics is stressed.

References

[1] Becker, D. E., and Smith, S. E.: A Chemical and Morphological Study of Normal Sheep Blood. Cornell Vet., *40*, (1950): 350-356.

[2] Bessey, O. A., Lowry, O. H., and Brock, M. J.: A Method for the Rapid Determination of Alkaline Phosphatase with Five Cubic Millimeters of Serum. J. Biol. Chem., *164*, (1946): 321-329.

[3] Bessey, O. A., Lowry, O. H., Brock, M. J., and Lopez, J. A.: Determination of Vitamin A and Carotene in Small Quantities of Blood Serum. J. Biol. Chem., *166*, (1946): 177-188.

[4] Burch, H. B., Bessey, O. A., and Lowry, O. H.: Fluorometric Measurements of Riboflavin and Its Natural Derivatives in Small Quantities of Blood Serum and Cells. J. Biol. Chem., *175*, (1948): 457-470.

[5] Goodland, R. L., Sealock, R. R., Scrimshaw, N. S., and Clark, L. C.: Interference with the Ultramicro Ascorbic Acid Method of Lowry, Lopez and Bessey. Science, *109*, (1949): 494-495.

[6] Lowry, O. H., and Hunter, T. H.: Determination of Serum Protein Concentration with a Gradient Tube. J. Biol. Chem., *159*, (1945): 465-474.

[7] Lowry, O. H., Lopez, J. A., and Bessey, O. A.: Determination of Ascorbic Acid in Small Amounts of Blood Serum. J. Biol. Chem., *160*, (1945): 609-615.

[8] Maynard, L. A.: Physiological Approach to Livestock Nutrition Problems. J. Anim. Sci., *1*, (1942): 179-188.

[9] Quaife, M. L., Scrimshaw, N. S., and Lowry, O. H.: A Micromethod for Assay of Total Tocopherols in Blood Serum. J. Biol. Chem., *180*, (1949): 1229-1235.

[10] Russoff, L. L., and Piercy, P. L.: Blood Studies of Louisiana Dairy Cows. II. Calcium, Inorganic Phosphorus, Hemoglobin Value, Erythrocyte Count, Leukocyte Count and Differential Leukocyte Percentages. J. Dai. Sci., *29*, (1946): 831-838.

[11] Scrimshaw, N. S., Goodland, R. L., and Hutt, F. B.: Variations in the Ascorbic Acid Blood Levels of Hens. Poult. Sci., *28*, (1949): 45-51.

[12] Squibb, R. L., Fuentes, A., Guzmán, M., Aguirre, F., and Scrimshaw, N. S.: Twelve Constituents of the Blood Stream of 3 herds of Purebred Dairy Cattle in Guatemala. Unpublished manuscript, Instituto Agropecuario Nacional, Guatemala City, Guatemala.

[13] Squibb, R. L., Salazar, E., Scrimshaw, N. S., and Guzmán, M.: Growth of Pigs Fed Corn and Banana Rations and the Effect of Supplementing These Rations with Vitamin Concentrate and Aureomycin. J. Anim. Sci. To be published.

[14] Wintrobe, M. M.: Clinical Hematology. 2nd ed. Lea and Febiger, Philadelphia, Pa. 1946.

[15] Work, S. H.: Animal Husbandry in the Caribbean Area. J. Anim. Sci., *6*, (1947): 195-202.

Effect of Herpes Simplex Virus (Strain P38) in the Cave Bat (*Myotis Lucifugus*)

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THE VIRUS of herpes simplex (strain P38) employed in this study was furnished by the American Type Culture Collection of Washington, D. C. This virus, originally obtained from Dr. S. Flexner and D. K. Olitsky, was isolated by the above workers directly in rabbits which had previously been inoculated intracerebrally with vesicular fluid.[1, 2] The virus was maintained in rabbits for eleven years by intracerebral passage of infected rabbit brain and was also carried through 14 serial passages in mice by intracerebral inoculation at the laboratory of Dr. T. F. McNair Scott in the Children's Hospital of Philadelphia. On receipt at this laboratory, the virus had been passed 14 times in Swiss albino mice and the infected mouse brain was suspended in neutral glycerin. This material was passed once intracerebrally in Swiss albino mice at this laboratory before the initiation of the present experiment.

EXPERIMENTAL

Virus-bearing-brain material from the fifteenth intracerebral mouse passage was ground in a mortar with alundum and diluted to a 10 per cent suspension with sterile physiological saline. This material was used as inoculum for the present experiment and titrated $10^{-3.5}$ intracerebrally in Swiss albino mice. The present experiment was carried out in the following manner: Fifty-three cave bats were divided into 11 groups, with 5 bats in each group, except 3 bats in the intracardiac group. Each group except for the normal controls was administered the virus by one of the following routes of inoculation: intracerebral, intraperitoneal, intradermal, intramuscular, intralingual, intratesticular, and intracardiac injection, and oral, rectal, and intranasal instillation. Three-hundredths milliliter of the above inoculum was given to the bats in the intracerebral and intranasal groups. The animals in each of the remaining groups were inoculated with 0.06 ml. of the virus suspension. All groups, after exposure to the virus by the above mentioned routes were observed twice daily for symptoms characteristically found in mammals infected with herpes simplex virus. Those bats displaying nervous symptoms and paralysis were destroyed and the brains removed aseptically and stored in a CO_2 deep freeze vault. After bats from all groups were destroyed, the brains were thawed and those from each group were pooled and ground to a 20 per cent suspension with physiological saline. The 5 bats in the control group appeared normal and were discarded. Suspensions of brain material harvested from bats inoculated with herpes simplex virus were shown to contain this virus by the following procedure: Unvaccinated Swiss albino mice and Swiss albino mice previously immunized against herpes simplex virus were injected intracerebrally with the above suspensions. Immunization was accomplished by intradermal and intramuscular injection of high dilutions of the original inoculum over a period of six weeks. Between three to four days post-inoculation, unvaccinated Swiss albino mice inoculated with suspensions of bat brain from the intratesticular, nasal, and intracerebral groups showed typical symptoms of herpes simplex. The immunized Swiss albino mice remained normal during a fourteen-day observation period.

TABLE 1—Response of the Cave Bat (Myotis Lucifugus) to Herpes Simplex Virus (Strain P38)

Route of inoculation	No. bats exposed	No. showing symptoms	Min. to max. incubation period * (hours)
Intraperitoneal	5	0	0
Rectal	5	0	0
Intradermal	5	0	0
Intracardiac	3	0	0
Intramuscular	5	0	0
Intralingual	5	0	0
Intratesticular	5	3	72
Intranasal	5	5	48
Oral	5	0	0
Intracerebral	5	5	48
Normal Controls	5	0	0

* Incubation period = time between exposure to virus and onset of symptoms.

RESULTS

Table 1 shows the response of the cave bat to exposure of herpes simplex virus by various routes. The incubation period in cave bats was between forty-eight and 72 hours.

From the Live Stock Sanitary Service Laboratory, Maryland State Board of Agriculture, University of Maryland, College Park.

Professor of veterinary virology (Reagan), virologist (Day), assistant electron microscopist (Sansone), and director (Brueckner).

SUMMARY

A strain of herpes simplex virus (strain P38) initially isolated in rabbits inoculated intracerebrally with vesicular fluid and carried eleven years by intracerebral passage of infected rabbit brain and also carried 15 serial passages in mice by intracerebral inoculation has been successfully transmitted to the cave bat *(Myotis lucifugus)* by the following routes of exposure: intracerebral, intratesticular, and nasal. Normal bats not exposed to the virus and employed as room controls appeared normal throughout the duration of this experiment. The virus contained in the brains of bats of the intracerebral, intratesticular, and nasal groups was confirmed to be herpes simplex virus by intracerebral injection into unvaccinated Swiss albino mice, and into Swiss albino mice vaccinated against herpes simplex virus (strain P38). The vaccinated mice showed no nervous symptoms, while the unvaccinated mice inoculated with bat brain material from intratesticular, nasal, and intracerebral groups showed symptoms characteristically found in animals infected experimentally by intracerebral injection with herpes simplex virus.

References

[1] Flexner, Simon: IV. Recurring Strains of Herpes Virus. J. Expti. Med., ..., (1928): 9-22.

[2] Flexner, Simon, and Amoss, Harold L.: II. Herpetic Strains of Encephalitogenic Virus. J. Exptl. Med., *41*, (1925): 233-244.

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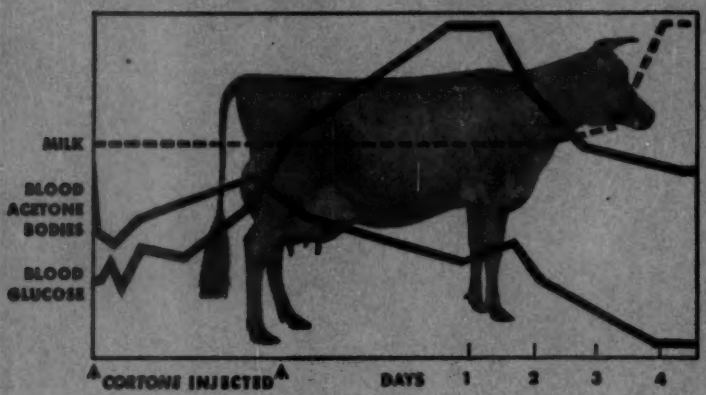

For Dramatic Relief of Bovine
KETOSIS

A single intramuscular injection of CORTONE Acetate *Veterinary* often effects complete remission of symptoms in both pre- and postpartum ketosis of dairy cows, in many instances after other forms of therapy have failed. Blood sugar levels have returned to normal in twenty-four hours, and milk production has been restored to normal in as little as three days.

Paralleling the dramatic results achieved by CORTONE in human medicine, CORTONE Acetate *Veterinary* also has been found effective against rheumatoid arthritis and inflammatory conditions of the eyes and skin. Its field of usefulness in animals continues to broaden as research progresses.

CORTONE Acetate Veterinary is available through a number of veterinary pharmaceutical manufacturers, in addition to pharmacies. Use is restricted to the veterinary profession.

CORTONE is the registered trade-mark of Merck & Co., Inc. for its brand of cortisone.

MERCK & CO., INC.
Manufacturing Chemists
RAHWAY, NEW JERSEY

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